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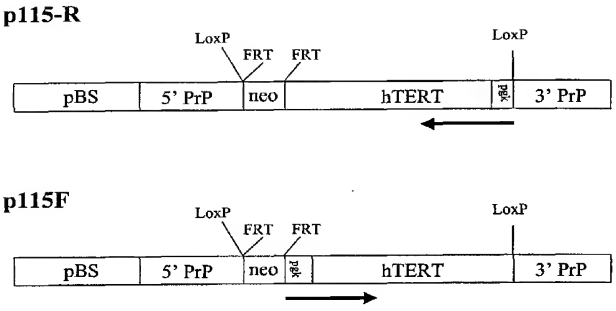

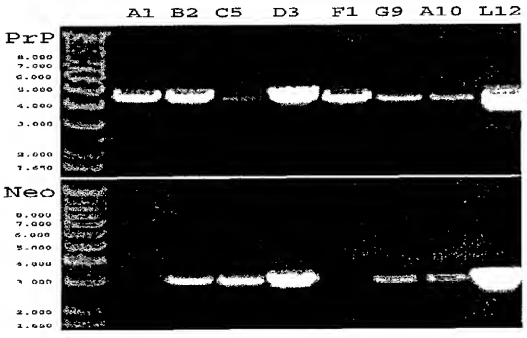

(57) Abstract: This disclosure provides a system for creating cloned cells and embryos that are genetically modified. Cells are treated to increase expression of telomerase and potentially extend replicative capacity. One or more genetic modifications is made to inactivate a gene or confer desirable features, growing and selecting the cells as needed. The modified nucleus can then be transferred to a suitable recipient cell, which can then be used to grow an embryo with the conferred attributes. This technology makes it possible to create embryos, animals and embryonic cell lines with multiple genetic modifications, including homozygously inactivated genes and gene substitutions.

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USE OF TELOMERASE REVERSE TRANSCRIPTASE TO CREATE HOMOZYGOUS KNOCKOUT ANIMALS

TECHNICAL FIELDS

This invention relates to the field of nuclear transfer between cells and animal cloning. The invention also relates generally to the field of cell senescence and increasing replicative capacity using telomerase reverse transcriptase.

RELATED APPLICATIONS

This application claims priority benefit of U.S. provisional patent application 60/277,749, filed March 21, 2001, pending. The priority application is hereby incorporated herein by reference in its entirety.

BACKGROUND

Animals were first cloned from adult cells by Keith Campbell and Ian Wilmut at the Roslin Institute, U.K. This seminal work has been described in U.S. Patent 6,147,276, International Patent Applications WO 97/07669 and WO 97/07668, and in Wilmut et al., Nature 385:810, 1997. The technique involves transferring the nucleus of a cell from the animal to be cloned into a suitable recipient cell. It is thought that the recipient cell causes the genes in the nucleus to be expressed in such a way that a program of embryonic development begins anew. The embryo is then implanted into a surrogate carrier animal for gestation into a viable offspring.

Subsequent to this teaching, other scientists have succeeded in cloning from adult cells. U.S. Patent 5,994,619 reports production of chimeric bovine or porcine animals using cultured inner cell mass cells. U.S. Patent 6,011,197 relates to a method for cloning cows by reprogramming non-embryonic bovine cells using leukocyte inhibitory factor (LIF) and fibroblast growth factor (FGF), then transferring the nucleus into an enucleated oocyte.

International Patent Publication WO 99/21415 reports nuclear transfer for production of transgenic animal embryos. WO 99/05266 and WO 00/52145 propose trans-species nuclear transfer, using bovine oocytes as the recipient cell for a nucleus taken from the donor cell of a different species. WO 99/36510 reports efficient nuclear transfer using fetal fibroblasts. WO 00/25578 proposes a cloning method in which an oocyte is chemically enucleated by exposure to a compound that destabilizes a meiotic spindle apparatus in the recipient cell. WO 00/31237 and WO 99/46982 outline methods for cloning pigs. WO 00/74477 propose a process for animal cloning in which somatic cells denatured by heating are transferred into enucleated metaphase II oocytes. WO 01/00795 describes surgical methods useful for obtaining oocytes from cows for cloning.

Loi et al. (Reprod. Nutr. Dev. 38:615, 1998) discuss embryo transfer and related technologies in sheep reproduction. Wells et al. (Biol. Reprod. 57:385, 1997) report production of cloned lambs from an established embryonic cell line. Liu et al. (Mol. Reprod. Dev. 47:255, 1997) discuss the effect of cell cycle coordination between nucleus and cytoplasm and the use of in vitro matured oocytes in nuclear

transfer in sheep embryos. Campbell et al. (Nature 380:65, 1996) report sheep cloned by nuclear transfer from an established cell line.

There is considerable promise in this field for cell therapy and adaptive agriculture. Until the technique of nuclear transfer was developed, genetically modified livestock were made by pronuclear injection (Clark et al., Transgenic Res. 9:263, 2000). Using this methodology, the nucleus of an embryonic cell can be transfected to place a new recombinant gene into the genome of the animal. The new transgene can have any one of a number of desired effects — such as causing secretion of a therapeutic protein into milk, which can then serve as a bioreactor for commercial production (A.J. Clark, Biochem. Soc. Symp. 63:133, 1998, and J. Mammary Gland Biol. Neoplasia 3:337, 1998).

The discovery that animals can be cloned by nuclear transfer from cultured somatic cells provides a new avenue for making animals with a modified genome.

SUMMARY

This disclosure provides a system for facilitating the creation of cloned cells and embryos that have been genetically modified. Cells are obtained that are suitable for nuclear transfer, and treated to increase expression of telomerase and potentially extend replicative capacity. One or more genetic modifications can then be made to confer desirable features, growing and selecting the cells as needed, which is facilitated by the effect of telomerase on the cells. The modified nucleus is then transferred to a suitable recipient cell, which can then be used to grow an embryo with the conferred attributes.

One embodiment of the invention is a method for producing a vertebrate cell with an altered genome by increasing telomerase activity in the cell at some point in the process of altering the genome.

Using this technique, multiple alterations to the genome are possible, including alterations to one or both alleles of a particular gene, or introducing transgenes at random locations, in any desired combination. Methods are provided in this disclosure to inactivate or replace the encoding region of a particular gene by homologous recombination or some other technique, and then selecting targeted clones by combinations of drug selection, mRNA analysis, or phenotype-based separation. Throughout this disclosure, it is understood that the altered cell can be expanded at any point in the process, and that multiple alterations to one cell is equivalent to alterations made sequentially to progeny of the cell.

Telomerase activity can be increased by expressing an encoding region for telomerase reverse transcriptase in either a transient or permanent fashion. If the TERT sequence is integrated into the genome in an expression cassette or as part of a targeting vector, it can later be removed by flanking the sequence with recombinase recognition sites.

Another embodiment of the invention is a method for producing a chimeric cell by nuclear transfer. A genetically altered donor cell is prepared according to the invention, and the nucleus is then transferred to a recipient cell suitable for chromatin remodeling or reprogramming, thereby permitting an embryo or pluripotent stem cell to be obtained. An embryo produced by this procedure can be used to birth a vertebrate animal by implanting into a suitable carrier. A major advantage of this strategy is that several genetic alterations can be produced in the embryo or animal in a single generation. The techniques of this invention may be brought to bear on any vertebrate species, amongst which livestock species such as sheep, cows, and pigs are exemplary.

Other embodiments of the invention are cells, embryos, and animals produced according to these methods. The cell, embryo or animal may have a normally expressed diploid gene which is inactivated or contains an artificially introduced genetic alteration. Where different targeting vectors are used, the gene may have a different alteration on each allele. A further embodiment of the invention is a cell line that has an increased level of telomerase activity or TERT expression, elected for high relative frequency of successful genetic targeting and is suitable as a donor for nuclear transfer.

Also embodied in the invention is a vector for homologous recombination in a eukaryotic cell, comprising a TERT encoding region for insertion into the genome. Homologous recombination is effected by way of other sequences in the vector that are highly homologous with genomic DNA to which it is targeted. The targeting site can be selected to inactivate an endogenous gene — such as the prion protein (PrP) gene, or the α(1,3)galactosyltransferase gene. This allows the cell to be targeted and telomerized at the same time, which improves replicative capacity of the cells and enhances genomic stability. The vector can have other features that enhance its use for nuclear transfer. For example, the TERT encoding region or a drug resistance gene can be is flanked on either side by recognition sites for a site-specific recombinase, to allow these sequences to be removed after genetic modifications are complete and the cell is being readied for nuclear transfer.

These and other aspects of the invention will be apparent from the description that follows.

DRAWINGS

Figure 1 is a map of plasmid pGRN145, which causes cells to express telomerase reverse transcriptase (abbreviated here as hTRT), the limiting component of telomerase activity in most mammalian cells. Transcription is under control of the myeloproliferative sarcoma virus (MPSV) promoter.

Figure 2 is a map of the adapted telomerizing plasmid PWpGB5. The MPSV promoter controls expression of the gene for telomerase reverse transcriptase (hTRT), followed by IRES sequences, and the puromycin selection gene. This entire region is flanked by *loxP* sequences, which allows the cassette to be excised from the genome after genetic manipulation and before expansion or nuclear transfer.

Figure 3 is a graph showing the growth of primary sheep fibroblasts transduced to express telomerase reverse transcriptase. Each line is a single clone, except BW6F2, which is the parental (untransfected) fibroblast line. O = telomerase-expressing clones; ▲ = telomerase-negative clones; ■ = clones that were telomerase-negative initially, but became positive later. All telomerase-negative clones became senescent towards the end of the growth curve, but cells with telomerase activity continued growing beyond 200 doublings.

Figure 4 is a photocopy of light micrograph of cloned sheep fibroblasts stained with X-gal for cell senescence. Telomerase negative clones are in the left panels, and contain >10% positive cells. Cells expressing hTERT, shown on the right, did not stain positively even when carried to ~256 doublings.

Figure 5 is a half-tone reproduction showing vectors used for gene inactivation in sheep fibroblasts. They are targeted to inactivate the α(1,3)galactosyltransferase (GGTA) and prion protein (PrP) genes by homologous recombination. Successful targeting creates PCR products and Southern

blot restriction fragments of different size. Results from clones shown on the right are for the unaltered wild-type cells (–), and cells with a gene knockout on one of the two alleles (+).

Figure 6 is a photocopy of a gel, showing results of targeting telomerized sheep fibroblasts with the α1,3GT targeting vector. Lanes: 1-5, PCR products using primers for the neo gene; Lanes 6-10, PCR products using primers for the α1,3GT sequence. Lanes 1, 2, 7, & 8 is clone B9; Lanes 3, 4, 8, & 9 is clone C9; Lanes 5 & 10 is a positive targeting control. Clone B9 shows successful inactivation of the *α1,3GT gene.*

Figure 7 is a map of the two promoter-less *neo* PrP gene knockout targeting vectors for targeting the sheep PrP gene. The vectors contain the hTERT gene in forward or reverse orientation, under control of the PGK promoter. These vectors can be used to inactivate the PrP gene and simultaneously telomerize the cells for further genetic manipulation and improved nuclear transfer efficiency.

Figure 8 is a half-tone reproduction of PCR analysis to identify cells in which the PrP gene was successfully targeted, shown by amplification product produced using *neo* specific primers and decreased *amplification product produced using PrP specific primers.*

Figure 9 is a half-tone reproduction of Southern analysis. In cells successfully targeted, a second band of 7.3 kb was detected, corresponding to the targeted allele.

Figure 10 is a half-tone reproduction of analysis for telomere restriction fragments (TRF) of targeted and non-targeted cell lines. Both the parental line (A1) and cells targeted without telomerization (L12) showed *shortened telomeres. However, the lines that were targeted and telomerized simultaneously retained long* TRF, indicating they retain replicative capacity and genomic stability.

DETAILED DESCRIPTION

In creating genetically modified animals, it is sometimes desirable to inactivate a gene that is normally expressed. This is a more difficult problem than turning on expression of a new gene — which can be accomplished by placing a single copy of the new gene into any transcribable site in the genome of the embryonic cell. To turn off expression of an endogenous gene, a specific locus in the genome must be targeted for genetic alteration. Furthermore, the gene usually must be targeted on both alleles before the desired phenotype is attained.

The availability of techniques for cloning by nuclear transfer considerably enhances the opportunity for producing genetically modified animals. The somatic donor cell is established in culture, and subjected to genetic modification and selection. The nucleus from the selected cell is then transferred to a suitable recipient cell that initiates formation of the embryo.

In experiments where particular genes were targeted in sheep cells by homologous recombination described below in the Example section, it was found that cells successfully targeted on one allele according to standard techniques may be suitable for nuclear transfer at only a low frequency. *In order to obtain a phenotypic knockout, it would be necessary to bring the cloned animal to term, and* then breed animals carrying the knockout on one allele until a homologous knockout animal is obtained.

It has now been discovered that the frequency of obtaining genetically modified cells suitable for nuclear transfer can be improved by increasing the expression of functional telomerase. The presence of active telomerase was found to have a number of important effects:

1. It increases the replicative capacity of the cell sufficiently to extend the genetic manipulation process — which can now include more extensive cell selection, and/or multiple serial genetic modifications on the same cell line. This substantially improves
2. It was found to improve the frequency of obtaining cell lines that have been successfully targeted by homologous recombination. As illustrated below, the usual frequency of targeting events is typically less than 1 in 100 cells, with only 1 in 10 of those cells forming cell lines. The presence of active telomerase apparently enhances the outgrowth and recovery of selected cells.
3. Unexpectedly, it also appears to increase the frequency of successful nuclear transfer. When the nucleus of one cell is transferred to a suitable recipient, active telomerase can in certain circumstances improve the probability that the reconstituted cell can be activated to grow into an embryo. This may be attributable to an effect of telomerase on enhancing or facilitating the chromosome remodeling that occurs during reprogramming of the nucleus in the recipient cell.

These effects need not all be present or understood in order to practice the invention, but may assist the reader in understanding the approach being taken.

The invention is particularly powerful to achieve more than one genetic modification in a single generation. In particular, homozygous knockout cells can be created in which the gene is modified either simultaneously or sequentially on both alleles. For example, the gene can be targeted with one vector comprising a drug resistant gene, and selected using the corresponding drug. The gene is then targeted with a second vector comprising a second drug resistant gene, and selected using the second drug. The surviving cell can be used to clone an animal that is modified on both alleles, without having to inter-breed to obtain the desired trait. In this illustration, the genome of the cell (and the cloned animal) will contain a different artificial genetic modification in each allele (i.e., the two different drug-resistant genes). This can be used, for example, to create homozygously inactivated genes, and genes that have been inactivated on one allele, and modified or substituted on the other.

The proliferative capacity is increased using telomerase to facilitate genetic modifications of this sort, but is typically not required subsequently. Where telomerase activity is increased by genetic transfection, this invention also provides for removing the transfecting gene after the other genetic modifications are complete. If the transfecting gene is integrated into the genome of the cell, it can be flanked with recombination sites and removed at an appropriate time by site-specific recombination.

Definitions

For purposes of this disclosure, an "endogenous" gene refers to a genetic locus that naturally occurs in the cell of a vertebrate species, in its normal context in an unaltered form. The gene may or may not include one or more encoding regions, one or more control elements, and internal or flanking untranscribed or untranslated regions. An endogenous gene element (such as a promoter) that is part of the endogenous gene can remain functional in a modified gene (for example, by linking to a new encoding region).

A "naturally.expressed" gene is capable of being transcribed into a functional gene product (such as a biologically active protein or RNA molecule) in at least one cell type of an animal having it in its genome.

An endogenous gene in a cell or animal is said to be "modified" when the DNA sequence of the gene has been modified by recombinant means to alter the molecular or biological function of the gene or gene product in some measurable way.

An endogenous gene in a cell or animal is said to be "inactivated" when it is rendered incapable of transcribing a functional protein. For example, an inactivated gene may be missing necessary transcription or translation control elements, it may be lacking an essential part of the protein encoding region, or the encoding region may be placed out of phase. In another example, the gene may be interrupted by an inserted sequence, or mutated in such a way as to interfere with transcription or translation of the gene product. In a third example, the inactivated gene may produce a translation product that has been altered in such a way that it lacks important enzymatic activity of the native gene product. A gene is also "inactivated" when the normal encoding region is switched with an encoding region for a different gene product with a different biological function.

In the descriptions of genetic modification and inactivation in this disclosure, it is understood that changes to the genome of a cell are inherited by progeny of the cell, unless further genetic manipulation occurs. Thus, it is possible to select the modified cells, let them proliferate, and then make a subsequent modification to the progeny. A sequence of genetic modifications made to cell and its ancestors are considered equivalent to making all the modifications to the same cell, unless explicitly directed otherwise.

A cell is said to be "transfected", "genetically transformed", or "genetically altered", when the cell has been introduced with a recombinant polynucleotide, or is the progeny of such a cell that has inherited the polynucleotide. The alteration may (but need not) be integrated into the genome of the cell. Non-limiting examples include the following: 1. A cell containing a vector with a sequence encoding a protein of interest, capable of causing the protein to be expressed by the cell on a transient or inheritable fashion; 2. A cell containing a genetic construct for targeting an endogenous gene (whether or not the gene has been successfully targeted); and 3. A cell containing a genetic modification introduced by recombinant means.

The genetic alteration is said to be "inheritable" if progeny of the altered cell has the same alteration. Determination of whether the genetic alteration is inheritable can be made by detecting presence of the polynucleotide template (e.g., by PCR amplification), or by detecting a phenotypic feature (such as expression of a gene product or effect thereof) that depends on the genetic alteration to be manifest.

An "alteration to the genome" of the cell refers to a change in sequence of chromosomal DNA (a deletion, insertion, or mutation) introduced by artificial manipulation of the cell, particularly by recombinant DNA technology. The change will be inheritable by progeny of the cell acquiring the altered chromosome, by chimeras made by transferring the nucleus of the cell to a suitable recipient cell, and by embryos or animals grown from them.

A cell is described as "telomerized" if it has been treated to increase the expression of telomerase reverse transcriptase (TERT) and/or functional telomerase activity by any suitable means beyond the level usually expressed by cells of the same type in the same environment. Methods for telomerizing cells are illustrated in a later section of this disclosure. The term also applies to progeny of the originally treated cell that have inherited the ability to express telomerase at an elevated level.

The terms "polynucleotide" and "oligonucleotide" are used interchangeably to refer to a polymeric form of nucleotides of any length. Included are genes and gene fragments, mRNA, cDNA, plasmids, vectors, synthetic nucleic acids, targeting constructs, nucleic acid probes, and primers.

A "control element" or "control sequence" is a nucleotide sequence involved in an interaction of molecules that contributes to the functional regulation of a polynucleotide, such as replication, duplication, transcription, splicing, or translation. Transcriptional control elements include promoters and enhancers.

The term "embryo" as it is used in this disclosure refers to an organism developing from a fertilized ovum or its equivalent generated by nuclear transfer technology. This includes an embryo growing *in utero*, and an early embryo growing in tissue culture before engrafting into a carrier. The terms "engrafting" or "transplanting", in reference to embryo manipulation, refer to any known process for artificially introducing one or more embryos into the uterus of a female animal.

The term "tissue" refers to a heterogeneous collection of cells responsible for maintaining one or more physiological functions. Of interest for certain embodiments of this invention are organs suitable for transplantation, such as a whole kidney; however, the term also includes organ fragments and other embodiments, such as a piece of connective tissue, or a collection of cells in a medical support device.

This invention can be practiced on cells of any vertebrate animal, such as a member of an avian or mammalian species, including but not limited to domestic animals, non-human primates, humans, agricultural livestock, and vertebrates suitable for growing biological compounds or tissue for human therapy.

General Techniques

For further elaboration of general techniques useful in the practice of this invention, the practitioner can refer to standard textbooks and reviews in cell and molecular biology, tissue culture, embryology, and veterinary and human medicine.

Methods in molecular genetics and genetic engineering are described generally in the current editions of *Molecular Cloning: A Laboratory Manual*, (Sambrook et al.); *Oligonucleotide Synthesis* (M.J. Gait, ed.,); *Animal Cell Culture* (R.I. Freshney, ed.); *Gene Transfer Vectors for Mammalian Cells* (Miller & Calos, eds.); *Current Protocols in Molecular Biology* and *Short Protocols in Molecular Biology, 3rd Edition* (F.M. Ausubel et al., eds.); and *Recombinant DNA Methodology* (R. Wu ed., Academic Press). Reagents, cloning vectors, and kits for genetic manipulation referred to in this disclosure are available from commercial vendors such as BioRad, Stratagene, Invitrogen, and ClonTech.

Texts that describe reproductive techniques and embryo transfer in animals include *Manual of the International Embryo Transfer Society: A procedural guide and general information for the use of embryo transfer technology emphasizing sanitary procedures*, 3rd ed. (Stringfellow et al., Savoy, IL: International Embryo Transfer Society, Savoy IL); and *Embryo transfer in farm animals: A review of techniques and applications* (K.J. Betteridge, ed., Agriculture Canada Monographs No. 16, Ottawa, 1977).

Increasing telomerase activity in the nuclear donor

Donor cells for genetic manipulation according to this invention are typically nucleated cells of the desired species with a germ line genotype, selected to be easily maintained in culture. Exemplary are

primary fibroblast cells, which are relatively easy to prepare for most species. For example, cells are collected from sheep or pig fetuses at about 35 days of gestation, and subjected to mild trypsin/EDTA solution, then cultured in a suitable culture medium. Except where explicitly directed otherwise, the techniques of this invention can be applied to any cell type without restriction, including embryonic cells, primary cells from a fetus, offspring, or adult, and established cell lines from any vertebrate.

The replicative capacity of the nuclear donor cell is increased by increasing telomerase activity. This assists the cells in maintaining telomere length, thereby expanding the replicative capacity (the number of cell doublings possible before reaching the Hayflick limit and entering crisis). Typically, telomerase activity is modified before inactivation of the target gene, but such modifications are also permitted at a later stage in the procedure.

Increasing telomerase activity can be accomplished by a number of strategies, including but not limited to the following:

a) genetically altering the cell with a nucleotide having an encoding region for telomerase reverse transcriptase (TERT);

b) artificially placing TERT protein or telomerase holoenzyme into the cell;

c) altering TERT expression from the endogenous gene; or

d) altering expression of a telomerase related protein, thereby effectively increasing telomerase activity.

A convenient method for increasing telomerase activity is to genetically alter the cells so that they express TERT, which is usually the limiting component of telomerase enzyme expression. A TERT gene can be cotransfected with a gene for the telomerase RNA component, or a TERT can be selected that is compatible with the RNA component already expressed by the cell.

It has been discovered that when cells from large mammals such as sheep and pigs are genetically altered with human TERT, they express increased telomerase activity, which indicates that the hTERT gene product can combine with endogenous RNA component to create a functional enzyme. It is a hypothesis of this invention that combinations of mammalian TERT into the cells of other mammals will often be effective.

The human TERT gene sequence is provided in U.S. Patent 6,166,178, which also describes the use of TERT to increase replicative capacity of various cell types. The mouse TERT sequence is provided in International Patent Application WO 99/27113. Other publications with telomerase-related sequences include International Patent Application WO 98/21343 (Amgen); WO 98/37181 (Whitehead); WO 98/07838A1 (Mitsubishi); WO 99/01560 (Cambia), and U.S. Patent 5,583,016 (Geron Corp.). U.S. Patent 5,968,506 describes purified telomerase and methods for obtaining it. When TERT is referred to in this description, it is understood to mean a polypeptide comprising a TERT sequence from any mammalian, vertebrate, or other species, with or without alterations, so long as the polypeptide has telomerase activity when associated with telomerase RNA component, as measured by TRAP assay (described below) in the cell line being treated.

Typically, the vector will comprise a TERT encoding region under control of a heterologous transcription control element that promotes transcription in the intended undifferentiated or differentiated cell line. Sequences that can drive expression of the TERT coding region include viral LTRs, enhancers, and promoters (such as MPSV, SV40, MoLV, CMV, MSCV, HSV TK), eukaryotic promoters (such as

β-actin, ubiquitin, elongation factors exemplified by EF1α, and PGK) or combinations thereof (for example, the CMV enhancer combined with the β-actin promoter). Expression of a marker gene can optionally be driven by the same promoter that's driving the TERT gene, either as a separate expression cassette, as part of a polycistronic transcript (in which the coding regions of TERT and the marker gene are separated by an IRES sequence, allowing both individual proteins to be made from a single transcript driven by a single promoter), or as part of the same cassette (a fusion between the coding regions of both TERT and the marker gene, producing a protein that provides the functions of both TERT and the marker gene). Transfection and expression of telomerase in human cells is described in Bodnar et al., Science 279:349, 1998 and Jiang et al., Nat. Genet. 21:111, 1999.

An alternative strategy is to use a vector that substitutes or supplements the promoter in the endogenous TERT gene with a regulatory control element (such as those listed above) that increase expression in the cultured cells. Further illustration of the general strategy of replacing promoters in endogenous genes can be found in U.S. Patent 6,063,630.

When the nucleus of the telomerized cell is transferred to another cell and used to produce a cloned animal or embryo, the tissue will contain alterations to the genome of the donor cell. The presence of a recombinant TERT gene in a donor cell may have other consequences. Accordingly, it may be desirable to provide a mechanism for removing or otherwise inactivating the recombinant TERT gene once the telomeres have been elongated but before nuclear transfer, or before cloned cells are used for another purpose.

This invention provides a mechanism by which the replicative capacity of the nucleus donor cell is enhanced with a telomerase gene during genetic manipulation and selection, but then is removed before nuclear transfer. The telomerase expression cassette is provided in a form that is capable of being passed down during replication, typically by integration into the genome, but adapted for subsequent excision.

This can be accomplished by flanking the TERT gene and/or the transcription control element on both sides with recognition sequences for a site-specific recombinase. Suitable are *lox* sites recognized by Cre recombinase (U.S. Patent 4,959,317), and *frt* sites recognized by Flp recombinase (U.S. Patent 5,929,301). Other site-specific recombinases include XerC (Becker et al., Curr. Microbiol. 32:232, 1996), XerD (Subramanya et al., EMBO J. 16:5178, 1997), xisF (Genes Dev. 8:75, 1994), and Int recombinase (Kolot et al., Mol. Biol. Reprod. 36:207, 1999; Tirumalai et al., Proc. Natl. Acad. Sci. USA 94:6104, 1997). An illustrative *lox* containing TERT vector is provided in Example 1.

Also contemplated are vectors in which a particular gene (such as a selectable marker) is flanked by one type of recombinase recognition site, and the TERT gene or control element is flanked with another type of recognition site. An example is the following:

5'arm – *loxP* – *frt* – neopA – *frt* – pGK promoter – hTERTpA – *LoxP* – 3'arm

This allows the drug resistance marker (*neo*) to be removed from the line after selection using the first recombinase (Flp), while retaining TERT. Further genetic manipulation can then be performed — for example, targeting the other allele of the same gene, possibly using the same vector and selecting for

neo again. After all manipulation is complete, the TERT encoding region can be removed using the second recombinase (Cre).

Another way of obtaining cells with genomic modifications that do not include TERT is to increase telomerase activity without integrating a TERT gene into the genome. For example, TERT can be transiently expressed using a suitable expression system such as adenovirus, or by introducing TERT protein (or the telomerase holoenzyme) directly into the cell. The TERT will be diluted out as the cell divides, but extension of telomeres in the parent cell should increase replicative capacity of the cell line by several doublings.

Another alternative is to upregulate TERT expression from the endogenous gene by upregulating expression of trans-activating transcriptional regulators. The TERT promoter contains a number of regulator recognition sequences, such as c-Myc, SP1, SRY, HNF-3ß, HNF-5, TFIID-MBP, E2F and c-Myb. See International Patent Publication WO 00/46355.

A further alternative is not to increase TERT expression, but enhance the effective activity of telomerase already present in the cell. This can be done in cells that have an endogenous level of TERT expression, such as in bone marrow progenitor cells and gonadal tissue. For example, TRF1 and TRF2 are proteins that bind to telomere repeats and regulate access of telomerase (Smogorzewska et al., Mol. Cell Biol. 20:1659, 2000). Decreasing expression of such factors may enhance the ability of telomerase to increase telomere length, thereby increasing replicative capacity of the cell.

Characterizing cells with increased telomerase activity

Evidence of increased telomerase expression can be obtained by a variety of techniques, including but not limited to determining gene transcript levels (for example, by Northern or RT-PCR analysis), protein expression (for example, by immunocytochemistry), or telomerase activity (for example, by primer extension assay). Extended lifespan or replicative capacity of the treated cells, while often desirable, need not be positively demonstrated for the invention to be put into practice, except where explicitly required.

Telomerase activity can be determined, for example, by TRAP assay (Kim et al., Science 266:2011, 1997; Weinrich et al., Nature Genetics 17:498, 1997), or other suitable technique (e.g., U.S. Patent 5,741,677). Evaluation of hTERT expression by RT-PCR or immunoassay can be done by standard methods, using the sequences disclosed in U.S. Patent 6,166,178. The following assay kits are available commercially for research purposes: TRAPeze® XK Telomerase Detection Kit (Cat. s7707; Intergen Co., Purchase NY); Telo*TAGGG* Telomerase PCR ELISAplus (Cat. 2,013,89; Roche Diagnostics, Indianapolis IN); and LightCycler Telo*TAGGG* hTERT quantification kit (Cat. 3,012,344).

If desired, the cells can also be characterized as to their replicative capacity. This can be determined by passaging cells in a culture environment that supports growth, and monitoring the number of cell doublings. Unmodified fetal fibroblasts will typically grow through a number of doublings until they reach the Hayflick limit, and then enter into senescence. As illustrated in Figure 3, cells with increased telomerase activity may grow through additional doublings, 10, 25, 100 or more (over 100 doublings for fetal cells; over 50 doublings for adult cells), and may grow indefinitely if TERT continues to be expressed.

The cells can also be characterized by their ability to undergo specific gene targeting. This is determined empirically, according to the purpose for which the cells will ultimately be used. For example, the cells are divided into subpopulations or cloned by limiting dilution, and each line is then sampled and treated using a targeting vector and subsequent drug selection. Selected cells are then expanded, and the number of expanded colonies is quantitated as a proportion of cells in the originally targeted population. A frequency of more than 0.2%, 0.5%, or 2% may be obtainable in certain circumstances.

The cells can also be characterized by their suitability as nuclear donors. This is also determined by empirical methods. For example, cell lines that have proved to have good replicative capacity and/or a high frequency of successful targeting are used as nuclear donors for nuclear transfer into a suitable recipient cell, for example, in the cloning of embryos, as described below. Lines can then be selected for a relatively high frequency of activation of the recipient cell after transfer, or for a relatively high frequency of viable embryo production. A frequency of 2% or 10% may be obtainable in certain circumstances.

Particular embodiments of this invention include cell lines selected for one or more of these attributes in any combination.

Genetically altering the target gene

The genome of the nuclear donor can be altered in any manner that is desirable to have in the recipient cell, or an embryo or tissue made from it. For example, a mutation can be introduced into a native encoding region that corrects a congenital defect or adds some desirable trait. A new encoding region can be integrated into the genome proximal to an endogenous gene element, such as a promoter that will cause it to be expressed in certain cells. Conversely, a new control element can be integrated proximal to an endogenous encoding region, to enhance or redirect its expression.

The techniques of this invention are particularly appropriate for creating gene knockouts, in which a particular gene is inactivated on one or both chromosomes. There is a variety of ways in which a gene can be inactivated. For example, a control element that regulates transcription (such as a promoter or transcription start sequence) can be altered or deleted. Alternatively, the gene can be adapted so that any mRNA that is produced is not translatable into the protein product. This can be effected, for example, by deleting or altering a translation control element, such as a ribosomal binding site or a translation initiation codon. Alternatively, the gene can be adapted so that any protein that is produced lacks the essential features of the endogenous gene product. For example, the encoding region can be interrupted with stop codons, the encoding region can be placed out-of-phase, or critical portions of the protein may be missing, such as a structural component or a signal peptide for secretion. In a further alternative, the gene can be adapted so that the encoded protein no longer has the specificity of the natural gene product — for example, because a change in enzyme activity or ligand binding specificity.

Efficient targeting of the gene typically entails use of a targeting vector, comprising nucleotide sequence identical or nearly identical to a portion of the gene of interest, linked to another structure capable of introducing the alteration. One such method uses homologous recombination, in which a DNA vector comprising homologous regions recombines at the targeted site, substituting its DNA sequence for that of the target. Cloned cells that have been selectively targeted can be identified by PCR amplification

of a sequence exclusive to the targeting vector, restriction analysis of the recombination site, or expression phenotype.

Generally it is more convenient to include a selectable marker in the targeting construct, so that targeted cells can rapidly be separated from untargeted cells. U.S. Patent 5,614,396 describes a method for obtaining a cell containing a desired sequence in the cell's genome, by using a targeting vector having two regions homologous to the targeting sequence, flanking a sequence that is to be inserted, and having a selectable marker. The DNA undergoes homologous recombination at the target site, and recombined cells are recovered under selective culture conditions.

Positive selection markers include the *neo* gene, selectable using G418 or kanamycin; the *hyg* gene, selectable using hygomycin; the *gpt* gene, selectable using xanthine, and hypoxanthine-phosphoribosyltransferase (HPRT), selectable using hypoxanthine. Negative selection markers include thymidine kinase (*tk*), selectable using acyclovir or ganciclovir; HPRT, selectable using 6-thioguanine; and cytosine deaminase, selectable using 5-fluoro-cytosine. Markers can also have an intrinsic label, like green fluorescent protein or β–galactosidase, which permit clones of targeted cells to be identified and selected. Another option is a gene that causes expression of a cell-surface antigen — for example, a transmembrane protein targeted to the plasma membrane, or a glycosyltransferase that causes formation of a surface oligosaccharide determinant. Cells that have incorporated the targeting vector will be selectable using an antibody or lectin specific for the surface determinant by a technique such as affinity adsorption or fluorescence-activated cell sorting.

For effecting homologous recombination, U.S. Patent Nos. 5,464,764 and 5,631,153 describe a double-selection strategy, in which two sequences homologous to the gene target flank a positive selection marker, and a negative selection marker is attached to the 3' terminal of the second flanking region. Homologous integration retains the positive selection marker, but eliminates the negative selection marker, whereas random integration usually retains both markers. Thus, by screening for both markers sequentially or together, cells that have been correctly targeted will be positively selected, and those that have been incorrectly targeted are selected out. U.S. Patent 5,789,215 reports the use of homologous recombination targeting vectors for modifying the cell genome of mouse embryonic stem cells. Other information of interest for homologous recombination targeting can be found in U.S. Patent Nos. 5,589,369 and 5,776,774.

Example 4 describes illustrative targeting vectors that are capable of inactivating the sheep gene for α(1,3)galactosyltransferase (α1,3GT) (SEQ. ID NOs:2 & 3) via homologous recombination. The vectors comprise flanking regions identical to the targeted α1,3GT sequence, one side being about 1 kb, the other being at least 1 or 2 kb, in either order. In between the flanking regions is a selectable marker such as *neo*, designed to replace one of the Exons in the α1,3GT coding sequence. The selectable marker genes are not provided with their own promoter, and require continued translation through the upstream α1,3GT sequence in order to be expressed. This helps the marker select for properly integrated vector, because vector inserted at a random site will probably not link the marker gene to a suitable promoter, and resistance to the selector drug will not be conferred.

As an alternative to homologous recombination, a target gene can be inactivated using triplex-forming oligonucleotides that induce intrachromosomal gene conversion (Luo et al., Proc. Natl. Acad. Sci. USA 97:9003, 2000; Barre et al., Proc. Natl. Acad. Sci. USA 97:3084, 2000). Other techniques and

reagents can be found in Inonue et al., J. Virol. 73:7376, 1999; Cole-Strauss et al., Science 273:1386, 1996; Hasty et al., Mol. Cell Biol. 11: 4509, 1991; and International Patent Publication WO 98/48005.

Examples 5 to 7 provide illustrations of the use of targeting vectors for inactivating endogenous genes for α1,3GT and prion protein (PrP). A suitable cell line is combined with the vectors in a culture medium, and the vectors are introduced into the cell. In the illustration, the vectors are introduced by optimized conditions of electroporation. The cells are cultured for a time in an appropriate medium for maintenance of the cells, during which time the recombination event should occur. The cells are then subjected to culture conditions that permit outgrowth of cells bearing the selectable marker from successful recombination.

After genetic manipulation has been completed and altered cells have been selected, inactivation of the gene can be confirmed by testing at the mRNA level or at the protein level. The nature of the genetic alteration can be determined by PCR amplification using primers bracketing the targeted recombination site, and characterizing the amplification product, or by Southern analysis. If the targeting vector contains a unique sequence, then correct integration can be confirmed using a primer specific for the inserted sequence. Production of amplification product of the predicted size in a PCR reaction confirms correct integration.

The extended replicative capacity of the nuclear donor cells of this invention facilitates production of cells with a single genetic alteration, inducible by any suitable method, such as those already described. It is now also possible to undertake multiple genetic modifications on the same cell (or its progeny), before nuclear transfer.

For example, the techniques of this invention make it possible to generate a nuclear donor cell in which both alleles of a diploid gene are inactivated, or otherwise modified.

One method for generating cells modified on both alleles is to use a single targeting vector in combination with a selection process that requires double integration. This can be accomplished, for example, by assaying for the silencing of a naturally expressed autosomal dominant gene product. For example, if the gene causes expression of a cell-surface determinant, then the cells can be targeted, and then selected for phenotypic expression of the determinant. A cell not expressing the determinant should be inactivated on both alleles. The double recombination event will be statistically rare, but the extended proliferative capacity of the cell population puts batch screening for such an event within the scope of routine experimentation.

Another method for generating cells modified on both alleles is to use two different targeting constructs. The constructs can be each created with different selection markers that facilitate screening for double integration. For example, the cell can be targeted with a first targeting vector containing a first drug resistance gene, and selected using the corresponding drug. After a round of proliferation, the progeny can then be targeted with a second vector containing a second drug resistance gene, and selected using the second drug. In a variation of this technique, both targeting constructs are used at once, and selection of doubly modified cells is performed in a medium containing both drugs. The use of two different targeting constructs for the same gene on the two different alleles generates a cell in which the diploid gene contains a different artificial genetic modification in each allele.

Many types of genetic modifications are possible using these techniques. Cells and cloned animals with a gene knockout can be generated by inactivating the gene on both alleles. Gene

modifications are possible in which both alleles are modified to change the encoding region, for example, to correct a congenital defect, or provide an improved trait patterned on another strain or species.

It is also possible to inactivate a gene and substitute another encoding region. For example, the first allele is inactivated using a targeting vector that inserts a drug resistance gene in place of the transcription start signal of the endogenous gene. Heterozygous knockouts are selected using the corresponding drug. The second allele is then targeted using a vector that inserts the substitute encoding region before or in place of the transcription start signal of the endogenous gene, but under control of the endogenous promoter. In this way, expression of the first encoding region will be phenotypically suppressed, and the substitute encoding region will be expressed in its place with a similar tissue specificity.

The increased proliferative capacity of the cells makes possible not just the multiple targeting of a single locus — but any type of genetic manipulation comprising multiple events. Superimposed on the modification of one or both alleles of one or more gene locus, the practitioner has the option of inserting one or more transgenes into the genome for expression of new gene products. With reflection upon these illustrations in the context of this disclosure, other embodiments of the invention will come readily to the mind of the skilled reader.

The timing of the telomerization step bears consideration in the context of these genetic manipulations. It is typically most convenient to increase telomerase activity in the cell before any further genetic manipulation takes place. This helps ensure that telomeres will be maintained at the same length as the parental cell throughout the genetic modification process. However, it is also possible to increase telomerase activity as an intermediate step in the process (say, after a first round of drug selection), or even after several genetic manipulations have been performed, in order to restore telomeres to an appropriate length. Also contemplated are strategies in which the TERT encoding region is included in a targeting vector used to inactivate an endogenous gene. In this way, telomerization of the cell occurs simultaneously with inactivation of one of the alleles. The vector can contain its own promoter controlling TERT expression, or the vector can insert the encoding region into the genome operatively linked to the endogenous promoter, providing the promoter is active in the cell type being used to generate the nuclear donor cell.

Once the desired genetic modifications have been made to the nuclear donor, the cell can be prepared for nuclear transfer. If TERT expression was increased by integrating a TERT encoding region into the genome, and if the cassette has been flanked with recognition sequences for site-specific recombination, as described in the previous section, then the cassette can be removed from the genome by introducing the corresponding recombinase into the cell.

Transient expression of the recombinase can be effected by transducing the nuclear donor cell with a suitable vector, such as an adenovirus or liposome-associated polynucleotide in which an encoding region for the recombinase is put under control of a heterologous promoter (such as those already listed) that is suitable for expression in the target cell. Also contemplated is a procedure whereby treatment with the recombinase is done after nuclear transfer, with the embryo (or its derivative cells) in culture.

Nuclear transfer and cloning

Once all the desired genetic manipulations have been performed, the donor cell can then be used for cloning. The nucleus is transferred into an enucleated recipient cell, such as an oocyte or other cell that is capable of developing into a fertile embryo after transfer and activation.

International Patent Application WO 97/07669 (Roslin Institute) describes quiescent cell populations for nuclear transfer. International Patent Application WO 97/07668 (Roslin Institute) describes inactivated oocytes as cytoplast recipients for nuclear transfer. For purposes of prosecution in the U.S., these patents and patent applications are hereby incorporated herein by reference in their entirety.

Nuclear transfer methods are particularly effective if the nucleus of the donor cell is quiescent, which can be achieved by culturing the donor cell in a serum-free medium (WO 97/07669). In an exemplary method, the nucleus of a donor cell is transferred into an oocyte that is arrested in the metaphase of the second meiotic division, and subsequently activating the reconstituted cell. Briefly, unfertilized metaphase II oocytes are collected as follows: Female animals are synchronized using progestagen sponges for ~14 days, and induced to superovulate with single injections of follicle-stimulating hormone on two successive days. Ovulation is induced with a suitable dose of gonadotrophin-releasing hormone or an analog thereof (e.g., ~8 mg GnRH Receptal™, Hoechst, UK) on the following day. The oocytes are recovered by flushing from the oviduct one day later, washed, and enucleated by treating with cytochalasin B and aspirating the nucleus using a glass pipette. Enucleated oocytes are then placed into contact with a single cell that acts as the nucleus donor.

Fusion of the donor nucleus into the enucleated recipient cell is effected by placing the couplet in a fusion chamber and aligning it between the electrodes. Electrical pulses are then applied to induce fusion, typically a low-voltage AC pulse for several seconds, followed by a plurality of very short high-voltage DC pulses. Following an incubation period, activation is induced by application of an additional electrical pulse. The reconstructed zygote is then cultured for a time before engrafting into a surrogate female. Further details and alternative procedures are described in the patent publications cited above.

Estrus in the surrogate female is typically synchronized artificially using a suitable combination of inducing agents. Cameron et al. (Aust. Vet. J. 66:314, 1989) discuss synchronization methods and other practical aspects for commercial embryo transfer in pigs. Blum-Reckow et al. (J. Anim. Sci. 69:3335, 1991) report experiments relating to transfer of pig embryos after long-term in vitro culture. Replacing medium every 12 h during culture improved survival, and pregnancy rate improved if the sexual cycle of recipients was 24 h behind that of the donor.

The embryos are introduced into the uterus of the recipient female using any suitable technique, including devices adapted for the purpose, or appropriate surgical methods. For example, U.S. Patent No. 4,326,505 describes surgical procedures for embryo transplants in animals, in which the uterine horn is positioned in the peritoneal cavity proximate to the vaginal wall, a cannula is inserted through the vaginal wall and into the uterine horn, and the embryo is introduced through the cannula. Non-surgical methods include using a suitable device to manipulate the injection port through the folds of the cervix to the bifurcation of the uterus. For example, devices and techniques for porcine non-surgical embryo transfer are reported by Li et al. (J. Anim. Sci. 74:2263, 1996). Wallenhorst et al. (J. Anim. Sci. 77:2327, 1999) describe the effect of transferring pig embryos to different uterine sites.

Use of cloned embryos

An embryo prepared according to this invention can be used for any desirable purpose, including but not limited to therapeutic cloning, cloning for agricultural purposes, production of embryo-derived cell lines and derivatives, and production of genetic knockouts and genetically modified animals to investigate gene function.

One potential use is the generation of animal tissue suitable for xenotransplantation. The main xenogeneic antigen causing rejection of animal tissues in humans is the cell-surface oligosaccharide determinant Galα(1,3)Gal. The epitope is made by α(1,3)galactosyltransferase, present in the cells of most mammals, but not humans (Joziasse et al., Biochim. Biophys. Acta 1455:403, 1999).

This invention provides a method for making animal tissue free of the Galα(1,3)Gal antigen by knocking out the α1,3GT gene on both alleles. The sheep α(1,3)galactosyltransferase cDNA sequences are provided in SEQ. ID NOs:2 & 3 of this disclosure, and the corresponding biological deposit. The pig α(1,3)galactosyltransferase cDNA sequence can be found in Strahan et al., Immunogenetics 41, 101 (1995) and GenBank Accession L36152; U.S. Patent 5,821,117; U.S. Patent 5,849,991; and International Patent Application WO 95/28412. The genomic organization of porcine α1,3GT was reported by Katayama et al. (Glycoconjugate J. 15:83, 1998). Example 1 below provides exemplary vectors with different drug resistance genes that can be used for sequential inactivation of the two α1,3GT alleles. Examples 5 to 7 illustrate how such vectors are used in telomerized animal cells suitable for nuclear transfer.

Also contemplated is modification of the animal tissue with other glycosyltransferase enzymes. In place of the Galα(1,3)Gal epitope on human cells, the N-acetyl lactosamine acceptor oligosaccharide is processed by an α(1,2)fucosyltransferase (α1,2FT). This enzyme makes the determinant Fucα(1,2)Galβ(1,4)GlcNAc (otherwise known as H precursor substance), present on most human cells, and the acceptor substrate for ABO blood group substance. Switching the α1,3GT gene in animal tissue to α1,2FT is believed to have advantages in preparing xenotransplant tissue. The tissue may further contain transgenes of the ABO blood group transferases.

Another potential use of this invention is to improve the safety of agricultural products. Creutzfeldt-Jakob disease is a fatal human neurodegenerative disease caused by prions. A variant form (vCJD) is thought to relate to the consumption of beef from animals affected with bovine spongiform encephalopathy ("Mad Cow Disease"). Scrapie is the corresponding spongiform disease in sheep and goats. The pathological characteristics of prion diseases include neuronal vacuolation, astrocytic gliosis, and amyloid plaques with filaments composed of prion protein. The gene for prion protein (PrP) is present in all species, and heavily implicated in disease pathology (Bolton et al., Science 218, pp. 1309-11 (1982); (Basler et al., Cell 46, pp. 417-28 (1986). However, the physiological role of PrP is uncertain, and it appears that mice can get along perfectly well without it (U.S. Patent 5,698,763).

This invention provides a technique whereby PrP expression is prevented in agricultural livestock, particularly sheep and cows. The sheep PrP gene sequence is provided in Goldmann et al., Proc. Natl. Acad. Sci. USA 87:2476, 1990 (SEQ. ID NOs:4 & 5). The bovine PrP sequence is provided in Goldmann et al., J. Gen. Virol. I72:201, 1991. Targeting vectors that disrupt the PrP encoding region

(Example 4) can be used in telomerized cells to produce nuclear donors that are homozygously inactivated at the PrP locus.

A further potential use of an embryo prepared according to this invention is the generation of stem cell lines. Human stem cells can be established from blastocysts and passaged in culture by known techniques (U.S. 6,200,806 and WO 99/20741). The stem cells can then be differentiated into specialized cells (such as cells of the hepatocyte or neural lineage) or their precursors, and used for such purposes as preparing therapeutic compositions for regenerative medicine, and testing the metabolic effects of potential medicaments. If telomerase activity is increased in the nuclear donor by integrating a TERT gene flanked by recombination sites, the gene can be removed before nuclear transfer, or after establishing a cell line from the embryo.

The examples that follow are provided by way of further illustration, and are not meant to imply any limitation in the practice of the claimed invention.

EXAMPLES

Example 1: Expression of hTERT in sheep nuclear donor cells

A vector containing an expression cassette for telomerase reverse transcriptase was found to increase functional telomerase activity and replicative capacity in sheep fibroblasts suitable for nuclear transfer.

Figure 1 is a map of plasmid pGRN145. It contains sequences encoding telomerase reverse transcriptase (abbreviated here as hTRT) with a consensus Kozak sequence downstream of the myeloproliferative sarcoma virus (MPSV) promoter. It also contains puromycin and hygromycin resistant gene sequences and allows drug selection of the transfected clones. SEQ. ID NO:1 is the nucleotide sequence of pGRN145.

Primary sheep fibroblast cell line designated BW6F2 (passage 6, obtained from a Black Welsh sheep) was transfected with linearized pGRN145. The cells were plated in 96 well plates, and selected using puromycin at 1 μg/mL. PCR screening with puromycin primers showed that all but one of the selected clones contained the vector sequence.

Fourteen of the clones were developed into cell lines. hTERT expression was measured in the cloned sheep fibroblasts by Western blot. Thirty μg cell lysate was separated by 7.5% SDS-PAGE, blotted onto nitrocellulose, and detected using antibody 1A4 (specific for hTERT) at 1:10,000, followed by goat anti-mouse IgG labeled with horse-radish peroxidase at 1:5000.

hTERT expression was also measured by immunocytology. Cells were grown in chamber slides and fixed with 4% paraformaldehyde. The cells were stained with 1A4 anti-hTERT antibody at 1:1000 dilution for 1 h, then with biotinylated secondary antibody at 1:500, and finally with streptavidin Texas Red™ at 1:200. Nuclei were counterstained with DAPI. hTERT protein was detected by immunocytochemistry in some cell lines but not others, correlating with hTERT production in the lines detected by Western blot.

Functional telomerase activity was measured by TRAP assay, and was found to be positive in 10 of these clones, compared with the original BW6F2 line.

Vector containing an excisable TERT cassette

The pGRN145 vector has been adapted to flank the hTERT encoding region with two *lox* sites for site-specific recombination and removal of the gene before nuclear transfer.

Figure 2 shows the design of the adapted targeting vector. It is an IRES bicistronic expression vector, comprising the MPSV promoter, followed by hTERT cDNA, IRES sequences, and the puromycin selection gene. This entire region is flanked by *loxP* sequences.

The hTERT vector was constructed as follows. A 0.85 kb fragment containing the SV40 small-t intron and polyadenylation signal was blunt-ligated into SalI site of pBluescript™ IIKS. A BlpI-NotI fragment of plasmid GRN145, containing MPSV promoter and hTERT cDNA was blunt-ligated into PstI site. An EcoRI-XbaI fragment from a plasmid containing IRES sequences and puromycin resistant gene, was blunt-ligated into HindIII site.

Finally, the NotI fragment (comprising the MPSV promoter, hTERT cDNA, IRES sequences, puromycin resistant gene and SV40 polyadenylation signal) was blunt-ligated into EcoRI-XhoI sites of a plasmid containing two *loxP* sites. The conjunctions have been sequenced to confirm the fidelity. This vector has been designated pWGB5 (SEQ. ID NO:6). An empty vector control was constructed by deleting the hTERT cDNA sequence from pWGB5 by EcoRI digestion and religation.

Example 2: Human TERT extends replicative capacity in non-human nuclear donor cells

In order to determine the replicative capacity of the cloned fibroblast cell lines derived in Example 1, the cells were passaged continuously using standard culture conditions. The cells were cultured in GMEM containing 10% fetal calf serum at 37°C, 5% CO_2 in continuous log phase.

Figure 3 shows the growth curves for these cells. Each line represents a single clone designation, except BW6F2, which is the parental (untransfected) line. The solid circles represent telomerase-expressing clones, and the solid triangles represent telomerase-negative clones. Open squares represent clones that were telomerase-negative initially, but became positive later. All telomerase-negative clones became senescent towards the end of the growth curve, as did the parental BW6F2 cells.

The clones expressing hTERT have been grown through at least 260 population doublings (PDs) and still grow like young cells. Cells transfected with a control plasmid without hTERT cDNA or the transfected cells not expressing hTERT grew less than 83 PDs. The parental cells only replicate through 127 PDs, when they become senescent.

Figure 4 shows staining of the cells with X-gal, which stains senescing cells blue. The cells were fixed in 2% formaldehyde/0.2%glutaraldehyde for 3-4 min, then stained with X-gal solution at pH 6. Telomerase negative clones are in the left panels; telomerase positive clones are in the right panels. Cells expressing hTERT showed no positively staining cells after they had been carried to ~256 PDs. In contrast, the cells transfected with the control vector showed over 10% positively staining cells when they had grown to less than ~50 PDs.

No evidence for malignant transformation

The hTERT expressing sheep fibroblasts were analyzed to determine whether or not the hTERT expressing cells showed signs of transformation to a malignant phenotype.

The cells were assessed for karyotype stability. Eight of the 10 telomerase-expressing clones showed normal karyotype when measured (passages 13 to 97).

The cells were also assessed for response to serum starvation. The cells were cultured in medium containing only 0.1% serum for 7 days, and assessed by FACS analysis (fixed in 70% ethanol and stained with propidium iodide). None of the telomerase-expressing clones was proliferating under these conditions. The cells were then recultured 24 h in 10% serum containing medium, and synchronized normally. When cultured for 3 days after reaching confluence, the cells also showed evidence of contact inhibition.

Telomere length was assessed by extracting DNA from cloned cells using a blotting assay. The DNA was digested with RsaI and HinfI, separated on 0.7% agarose, blotted onto a nylon membrane, and probed with ^{32}P-labeled $(TTAGGG)_3$ oligonucleotide.

It was found that clones with higher hTERT expression levels (detected by Western blot and immunocytochemistry using 1A4 antibody) maintained their telomere length, while clones with lower hTERT expression levels were typically the ones showing shortened telomeres.

A summary of results from these experiments is shown in Table 1.

TABLE 1: Characteristics of Telomerized Sheep Fibroblast Clones

Designation	PCR for puromycin gene	TRAP assay (telomerase activity)	Population doublings observed	Response to serum starvation	Contact inhibition	Karyotype
GRN 1-1	+	–	354	Normal (p54-56)	Normal (p72)	Normal (p14, p49)
GRN 1-2	+	– → +	289	Normal (p50)	Normal (p47)	Normal (p8, p35)
GRN 2-1	+	+	264	Normal (p50)	Normal (p43)	Normal (p13, p80)
GRN 2-2	+	– → +	294	Normal (p48)	Normal (p52)	Normal (p30) Abnormal (p90)
GRN 2-3	+	–	37[a]	*n.d.*	*n.d.*	*n.d.*
GRN 2-4	+	–	75[a]	*n.d.*	*n.d.*	*n.d.*
GRN 2-5	+	+	279	Normal (p54)	Normal (p46)	Normal (p12, p86)
GRN 2-7	+	+	314	Normal (p62)	Normal (p64)	Normal (p15, p97)

TABLE 1: Characteristics of Telomerized Sheep Fibroblast Clones

Designation	PCR for puromycin gene	TRAP assay (telomerase activity)	Population doublings observed	Response to serum starvation	Contact inhibition	Karyotype
GRN 2-8	+	+	318	Normal (p60)	Normal (p52-53)	Normal (p15)
GRN 2-10	+	+	166[b]	*n.d.*	*n.d.*	Abnormal (p13)
GRN 2-12	+	+	293	Normal (p50)	Normal (p51-53)	*n.d.*
GRN 2-13	+	+	258	Normal (p47)	Normal (p48-49)	Normal (p16)
GRN 2-18	–	–	83[a]	Abnormal (p18)	*n.d.*	*n.d.*
GRN 2-20	+	– → ?	113[b]	*n.d.*	*n.d.*	*n.d.*

[a] – Cells became senescent
[b] – Growth curve stopped

Expression of hTERT in pig nuclear donors

A primary fibroblast cell line was isolated from fetal pig carcass, and seeded into 25 cm^2 flasks at 2.5×10^5 cells in 10 mL Dulbecco's MEM containing 20% serum.

Lipofection with hTERT was performed as follows: 4.4×10^6 PF6C p3 cells were transfected with lipofectamine™ plus 30 μg pGRN145 at a ratio of 3:1 with 4 h incubation. Twenty-four hours after transfection, the cells were divided into 6 × 10 cm plates (7 $x10^5$ cells per plate). After 3 days, the cells were 95% confluent, and selection medium was added.

Electroporation was performed as follows: 4.6×10^6 PF6C p3 cells were washed and electroporated in prepared electroporation buffer (9 hypo-osmolar : 1 iso-osmolar). The Eppendorf™ electroporator settings were 300 v, 100 μs. Immediately after electroporation ,the cells were divided into 6 × 10cm plates (7×10^5 cells per plate). After 3 days, selection medium was added to each plate.

Sixteen days after transfection, established colonies of isolated cells were visible in the six lipofection plates. In comparison, in the electroporation plates only single cells were visible with no colony formation. Thirty seven colonies were picked by ring cloning and transferred to 12 well plates. . After 6 days, 10 colonies were confluent and transferred to a 25 cm^2 flask. Within 10 days, eight of these colonies were expanded up to a 75 cm^2 flask, frozen into six vials, and stored in liquid nitrogen.

When analyzed by immunocytochemistry using hTERT antibody 1A4, most clones showed cytoplasmic staining. One clone designated PF6C-T3C showed significant nuclear staining, similar to the cloned sheep cell line GRN2.2 (Example 1).

Example 3: Human TERT improves genetic stability in non-human nuclear donor cells

It has been suggested that the telomeres play an important role in genome stability (Hackett et al., Cell 106:275, 2001; Riha et al., Science 291:1797, 2001). To assess the effects of hTERT expression on the genome stability of the stably transfected sheep fibroblast, the cell lines transfected with the hTERT gene were analyzed for hTERT expression and activity, and cytogenetic analysis.

Telomere length was determined by telomere restriction fragment (TRF) Southern blot analysis as described previously (Harley et al., Nature 345:458, 1990). Briefly, 1-3 μg of DNA was digested with restriction enzymes *HinfI / RsaI* and fractionated by 0.7% agarose gel electrophoresis, then transferred onto a positively charged nylon membrane. The membrane was hybridized with $(TTAGGG)_3$ probe labeled with either γ-[^{32}P]ATP or digoxigenin. Signals were visualized by phosphorImager scan either directly (^{32}P labeled probe) or after antibody/substrate reactions (digoxigenin labeled probe). Mean TRF was calculated as described by Ouellette et al., J. Biol. Chem. 275:10072, 2000.

Expression of hTERT and endogenous sheep GAPDH was measured as follows. Cells were trypsinized and counted, then washed with PBS. Total RNA was extracted with RNAzol B and digested with DNase I. First strand cDNA was synthesized from 5μg of total RNA by reverse transcriptase in 20 μL volume with oligo $Pd(T)_{15}$. Each PCR was carried out with 5μl of the reverse transcription product and amplified for 26 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 45 sec using specific primers for human TERT or sheep GAPDH. Real time qRT-PCR primers and TaqMan probes were selected for hTERT and sheep GAPDH using Primer Express software (Applied Biosystems). Each TaqMan™ reaction was performed in quadruplicate. The hTERT mRNA copy number was calculated with a standard curve generated from hTERT cDNA plasmid at concentrations equivalent to 0.01 to 1000 copies per cell. Telomerase activity of cell extracts was analyzed by telomeric repeat amplification protocol (TRAP) assay as described previously (Kim et al., Nucleic Acids Res. 25:2595, 1997). Results are shown in Table 2:

TABLE 2: Telomere length, Telomerase Activity, and hTERT Expression

Cell clone	Mean TRF[a] (kb)	TRAP Assay[b]		Relative hTERT mRNA Expression[c]	hTERT mRNA per Cell
		PD = 0	PD > 200		
1-1	8.4	30%	48%	2.6	0.34
2-1	20.7	51%	46%	5870	375
2-5	21.0	39%	52%	1280	87
2-7	21.2	30%	52%	5250	345
2-8	10.8	52%	39%	8.1	0.58
2-12	14.6	60%	55%	9.3	0.65
2-13	6.0	38%	30%	1.0	0.08
BW6F2 (young)	20.9	0	–	0	0.02
BW6F2 (senescent)	11.3	–	0	not done	not done

[a] Mean TRF when telomere length is stabilized
[b] TRAP activity compared with human 293 cell line
[c] Measured by qRT-PCR, normalized with internal GAPDH level, and calculated relative to clone 2-13

The cell lines exhibiting high steady-state hTERT mRNA levels and detectable hTERT protein (2-1, 2-5 and 2-7) did not undergo telomere shortening and they fully maintained their telomere lengths. Line 2-8 and 2-12, which had low levels of hTERT mRNA and undetectable levels of hTERT protein, exhibited telomere shortening but their telomeres were stabilized with a mean TRF greater than 10 kb. Lines 1-1 and 2-13, with very low level of hTERT mRNA also exhibited telomere shortening but their telomeres shorten to an even greater extent and were only stabilized when the mean TRF was below 10kb.

For karyotype analysis, slides with metaphase spreads were stained in 5% Gurrs R66 Giemsa and then mounted. Results are shown in Table 3:

TABLE 3: Karyotype of hTERT-expressing sheep fibroblasts

Cell line	Population Doublings	Days in Culture	Number of Cells			Abnormalities
			50	50 (100%)	0	
1-1	333	36	50	50 (100%)	0	
	222	197	10	8 (80%)	2)20%)	SM
	318	279	30	11 (37%)	19 (63%)	Smet, DIC, Mar
	363	322	30	2 (7%)	28 (93%)	SubM, SM, LM
2-1	29	47	30	30 (100%)	0	
	231	285	30	30 (100%)	0	
	264	320	30	30 (100%)	0	
2-5	26	47	30	30 (100%)	0	
	68	95	30	30 (100%)	0	
	248	285	30	30 (100%)	0	
	279	320	10	29 (97%)	1 (3%)	LM
2-7	36	48	30	30 (100%)	0	
	281	281	30	30 (100%)	0	
	313	316	30	29 (97%)	1 (3%)	SubM
2-8	36	48	30	30 (100%)	0	
	228	231	10	10 (100%)	0	
	286	284	30	24 (80%)	6 (20%)	
	317	316	30	15 (50%)	15 (50%)	SM, SubM
2-12	18	32	10	10 (100%)	0	
	201	230	10	10 (100%)	0	
	256	279	30	29 (97%)	1 (3%)	Mar
	429	433	30	16 (53%)	14 (47%)	LM
2-13	21	49	30	30 (100%)	0	
	225	280	30	5 (17%)	25 (83%)	Mar, Ring, DIC, LM,
	266	322	30	0	30 (100%)	SM, LM, SM, SubM, Ring

DIC, dicentric; LM, large metacentric; Mar, marker chromosome; Ring, ring chromosome; SM, small metacentric; SubM, submetacentric

At the beginning of their proliferative lifespan, all hTERT transfected clones showed normal karyotype. However, after about 220 population doublings, clones 1-1 and 2-13, started to exhibit a high frequency of abnormal karyotype (20% or more cells). These abnormalities included abnormal sub-metrocentric, dicentric and ring chromosomes, which likely resulted from chromosomal end-end fusions. The frequency of these abnormalities increased with cell ageing. Lines 2-8 and 2-12 also started to exhibit chromosomal abnormalities after extended culture, although this occurred at later PDs than was

the case for lines 1-1 or 2-13. By contrast, cells 2-1, 2-5 and 2-7 essentially maintained a normal karyotype even at high population doublings, although very occasionally an abnormal karyotype was observed. The genomic instability of these cell lines was inversely correlated with the level of hTERT mRNA expression: high hTERT expressing lines, 2-1, 2-5, 2-7, showed no abnormal karyotype; low hTERT expressing lines 2-12 and 2-8, showed a low frequency of abnormality (3% and 20% respectively); and very low hTERT expressing lines 1-1 and 2-13 showed high frequencies of karyotypic abnormalities (63% and 83%, respectively).

These data lead to the conclusion that high levels of TERT expression are required to maintain the genomic stability of these lines. When lower levels of TERT are present, the telomeres shorten and are then maintained at a standard length determined directly by the level of TERT expression. Genomic instability in terms of the timing and degree of karyotypic abnormalities is inversely related to the level of TERT mRNA expression. Thus, TERT may provide genomic stability by performing functions beyond telomere elongation.

Example 4: Construction of targeting vectors

Vectors have been constructed to disrupt the α(1,3)galactosyltransferase (α1,3GT) gene and the prion protein (PrP) gene by homologous recombination.

Vector for targeting galactosyltransferase

The sequence of the sheep cDNA for α1,3GT is shown in SEQ. ID NOs:2 & 3. To develop genomic constructs, DNA was isolated from Black Welsh Mountain fetal fibroblasts, and a λDASHII phage library was constructed. Sau3A partially digested genomic DNA was dephosphorylated and ligated to compatible BamHI vector arms (Stratagene). The ligation products were packaged to produce recombinant phage, which were propagated on *spi* selective XL1-Blue-MRA(P2) bacterial cells. The resulting library had a complexity of 1.4×10^6 recombinants, and was subsequently amplified once. Six phage clones were isolated that spanned Exon-4, Exon-6-7 and Exon-9.

Recombinant phage designated B, C and G, have been deposited as a pooled sample with the National Collections of Industrial and Marine Bacteria Limited (NCIMB), Aberdeen, U.K, under Accession No. NCIMB 41056. The phage can be separated using the oligonucleotide probes 5'-GGGAGGAAGC GAAGGTGCA-3' (SEQ. ID NO:7), 5'-CTTGATGGGT TTATCCAGAA CA-3' (SEQ. ID NO:8) and 5'-TGATAATCCC AGCAGTATTC-3' (SEQ. ID NO:9), respectively. Each recombinant phage has also been deposited separately with the NCIMB under the following Accession numbers: Clone B, No. 41059; Clone C, No. 41060; and Clone G, No. 41061.

A targeting vector was designed directed towards Exon 4 of the sheep α1,3GT gene. The vector comprises two regions that are complementary to genomic sequence; a 1.2-kb 5' arm, which includes sequence from Intron 3 leading up to and including the start codon in Exon 4, and a ~9-kb 3' arm that initiates 1.5-kb into Intron 4, continuing to Intron 5. Separating these regions is *neo*^{R}-polyA sequence. After homologous recombination, the vector confers neomycin phosphotransferase resistance to the cells and deletes 1.5-kb of genomic sequence, including the first coding exon of α1,3GT gene. The entire cassette was cloned into pBlueScript™ for propagation in DH5α bacterial cells.

This vector was designated p0054. It was constructed by amplifying a truncated left arm (300bp, includes EcoRI site) (using primers 199001, 5'-ACGTGGCTCC AAGAATTCTC CAGGCAAGAG TACTGG-3', SEQ. ID NO:10; and 199006, 5'-CATCTTGTTC AATGGCCGAT CCCATTATTT TCTCCTGGGA AAAGAAAAG-3', with tail complementary to the start of *neo* coding sequence, SEQ. ID NO:11), and a *neo*-polyA sequence obtained from Stratagene (using primers 199005, 5'-CTTTTCTTTT CCCAGGAGAA AATAATGGGA TCGGCCATTG AACAAGATG-3', SEQ. ID NO:12, with tail complementary to left arm; and 199004, 5'-CAGGTCGACG GATCCGAACA AAC-3', SEQ. ID NO:13). These fragments were used to prime from each other to give a 1.2-kb fusion product. This was ligated to Intron 3 sequence, to extend the left arm, and to ~9-kb of 3' sequence to create the right arm, which initiates 1.5-kb into Intron 4, continuing to Intron 5.

Another promoterless vector is constructed, having the designation p0063. Instead of the *neo* drug resistance gene, it contains the *pac* gene that codes for puromycin N-acetyltransferase, permitting a second round of screening using a different drug. The *pac* sequence is available in plasmid pPUR from ClonTech. The oligonucleotide primers used to generate the 5'-*pac*-polyA fusion were, for the 5' region, 199001 (SEQ. ID NO:14) and 699002 (5'-GCGCACCGTG GGCTTGTACT CGGTCATTAT TTTCTCCTGG GAAAAGAAAA G-3', SEQ. ID NO:15), with tail complementary to the start of *pac* coding sequence; and, for *pac*-polyA, 699004 (5'-GAGAAAATAA TGACCGAGTA CAAGCCCACG GTGC-3' SEQ. ID NO:16), with tail complementary to left arm, and 699005 (5'-CTGGGGATCC AGACATGATA AGATAC-3' SEQ. ID NO:17).

Vectors for targeting Prion Protein

The plasmid designated p0036 for homologous recombination to inactivate the sheep PrP gene responsible for scrapie was constructed as follows. Phage corresponding to GenBank Accession Number U67922 (Lee et al., Genome Res. 8:1022, 1998) was isolated using a PrP coding sequence probe (ATG start codon to TGA stop codon). PCR of the 5'arm using the sheep genomic library was performed using primers 6F (19786) and 7R (22278) to give a 2.4kb fragment. This also engineered a SacI site to the 5'end of the vector, which allowed the final vector to be linearized before transfection. PCR of neo-pA (from pMC1-neo; Stratagene) sequence was performed using primers 10F and 8R to give a 0.9kb fragment.

Primers 7R and 10F, which were used to produce these products, also contained sequence corresponding to the start of the neo gene and to the end of the 3' arm, respectively. By incubating the 2.4kb and 0.9kb fragments together in a second PCR reaction, they primed from each other to produce the fusion product of 3.3kb. This product was cloned into SacI and SalI sites in pBluescript. To complete the vector, a ~3kb KpnI fragment (23721 to 26748) was cloned into the KpnI site of the above precursor vector.

The primers used were as follows: Primer 6F: CCGAGCTCG CCAATTTCAT GGCTGCAGTCACC (SEQ. ID NO:18). Primer7R: CGATCCCAT GATGACTTCT CTGCAAAATAAAG (SEQ. ID NO:19). Primer 10F: GAGAAGTCA TCATGGGATC GGCCATTGAACA (SEQ. ID NO:20). Primer 8R: TGCAGGTCG ACGGATCCGAA (SEQ. ID NO:21).

Example 5: Targeting the galactosyl transferase and PrP genes

Electroporation conditions were optimized using a β-galactosidase marker plasmid, pCMV-Sport-βgal (Gibco). Using a 0.4 cm cuvette with 3×10^5 cells (0.8mL, 6 μg plasmid DNA) and a setting of 250 μF : 400 Volts (Gene Pulser, BioRad), 10-30% of the surviving fibroblasts stained positive for β-gal expression.

For targeting the α1,3GT gene, 10, 25 or 100 μg of NotI linearized p0054 vector was mixed with $1x10^7$ early passage Black Welsh Mountain fetal fibroblasts and pulsed. Cells were grown on tissue culture plastic for 24 h before G418 was added at 300μg/mL. After 10-14 days, colonies were isolated.

For targeting the PrP gene, 10 to 100 μg of SacI linearized p0036 vector was mixed with $1x10^7$ early passage Black Welsh Mountain fetal fibroblasts (BW6F2) and pulsed at 250uF:400V. Cells were grown on tissue culture plastic for 24 h, and then G418 was added to the medium at 400μg/mL. After 10-14 days, colonies were isolated.

Site-specific recombination was detected by PCR amplification. Wild type and targeted α1,3GT alleles were detected using sense (399010, 5'-CAGCTGTGTG GGTATGGGAG GG-3'; SEQ. ID NO:22) and antisense (499006, 5'-CTGAACTGAA TGTTTATCCA GGCCATC-3'; SEQ. ID NO:23) PCR primers, yielding products of 2.8-kb and 2.2-kb, respectively. A second PCR screen with primers 399010 (SEQ. ID NO:22) and 399005 (5'-AGCCGATTGT CTGTTGTGCC CAGTCAT-3'; SEQ. ID NO:24) produced a fragment of 1.5-kb only in clones that were correctly targeted.

PCR amplification for wild type and targeted PrP alleles was performed using sense (Target F1, 5'-TTCAGTCGCT CTGTTGTGTC CCA-3'; SEQ. ID NO:25) and antisense (Target R1, 5'-AGCATCCCTC CTGCCTTCAG TTCTTC-3'; SEQ. ID NO:26) PCR primers, yielding products of 4.6-kb and 3.9-kb, respectively. A second PCR screen with primers Target R1 (SEQ. ID NO:26) and 399005 (SEQ. ID NO:24) produced a fragment of 3-kb only in clones that were correctly targeted.

For Southern blot analysis of the α1,3GT gene, probe fragments were generated as follows. Oligos 800-005 and 800-006 were annealed by reducing the temperature by 1°C every 5 min from 94°C to 4°C. 800-005 forward= GAT CCC AGC TGT GTG GGT ATG GGA GGG AAA GGC CAC CTG GGA AAT GGT TGG GTC TCA ATT GTA AAA GAC CAG CAT GCT TTC TGC TCT GAA CGG CGG AGC ACG TAG TTA GG (SEQ. ID NO:27); 800-006 reverse= GAT CCC TAA CTA CGT GCT CCG CCG TTC AGA GCA GAA AGC ATG CTG GTC TTT TAC AAT TGA GAC CCA ACC ATT TCC CAG GTG GCC TTT CCC TCC CAT ACC CAC ACA GCT GG (SEQ. ID NO:28). These oligos correspond to genomic sequence immediately 5' to the left arm of the aGT targeting vector. They were ligated together by virtue of the engineered BamHI overhang sequences on each end thus forming a concatamer containing three repeated oligo sequences. For PrP, the region from 16701 to 17151 of accession u67922 was amplified by PCR and used as a probe fragment. This lies 5' of the left arm of the PrP targeting vector.

Southern analysis was conducted as follows. 10 μg genomic DNA was digested with BamHI, electrophoresed, and transferred to Ambion Bright Star™ membranes, using the Southern Max™ system according to manufacturer's directions. Membranes were prehybridized in Ambion Ultrahyb™ solution at 42°C; then probe was added and the incubation continued at the same temperature overnight. Membranes were washed in buffers of increasing stringency at 42°C. Patterns of hybridization were detected using a BioRad phosphorimager. Conditions for PrP S. blots were performed in the same way except genomic DNA was digested with BglI restriction enzyme.

Figure 5 shows schematically the targeting of the α1,3GT and the PrP genes undertaken in primary sheep fibroblasts. Each targeting vector replaces part of the protein encoding region with a shorter sequence that interrupts the normal encoding region, and simultaneously inserts a drug selection gene, and alters the distance between restriction enzyme cleavage sites. Successfully targeted clones are detected as an altered PCR amplification product, or by a different restriction fragment on Southern blot analysis of BamHI digested DNA.

The frequency of site-specific recombination observed in these experiments is shown in Table 2:

TABLE 2: Gene Targeting Efficiency in Primary Sheep Fibroblast Cultures

Parental culture	Target locus	Drug resistant colonies	Targeting events detected	Colonies suitable for nuclear transfer
Black Welsh	α1,3GT	877	10 (1.1%)	0 (0%)
Black Welsh	PrP	533	55 (10.3%)	1 (0.2%)
Finn Dorset	α1,3GT	568	35 (6.2%)	2 (0.4%)

Cells were prepared for nuclear transfer by the method already described, namely serum starvation for 5 days prior to use as a donor. Nuclear transfer is typically conducted as follows. Oocytes are harvested from adult female breeding sheep treated with an analogue of gonadotrophin releasing hormone (Buserelin™, given 24 h after sponge removal). The oocytes are stripped of cumulus cells by triturating with a pipette and incubating with hyaluronidase. They are then enucleated by removing the first polar body and metaphase plate. A single targeted nuclear donor cell is introduced under the zona of each oocyte. The cell combination is subject to simultaneous electrofusion and activation (0.25 kV cm^{-1} AC for 5 sec. to align oocyte and donor cell, followed by 3 pulses of 1.25 kV cm^{-1} DC for 80 μsec to fuse and activate the reconstructed embryo). The activated cell is maintained in culture overnight at 39°C. Next day, the cells are embedded in agar chips to protect from macrophages, and then transferred to the ligated oviduct of a temporary recipient.

Estrous is controlled in the temporary recipient by treatment with intravaginal progestagen sponge for 11 to 16 days, with or with subcutaneous or intramuscular injection of 500 i.u. of PMSG. The timing brings the temporary recipients to estrus ~3 days before the oocyte donors. Cells are collected under general anesthesia using barbiturate followed by gaseous anesthetics. The reproductive tract is exposed by midventral laparotomy; placing ligatures of silk at each uterotubal junction, and embryos are transferred through the fimbriated end of the oviduct. The laparotomy is then closed, and a long-acting antibiotic is administered. The embryos are flushed from the temporary recipient after 6 days, and developing embryos are removed from the agar chip.

Blastocysts and morula are then transferred into the recipients that will carry the embryo to term. Estrus is controlled by treatment with an intravaginal progestagen sponge for 11 to 16 days, bringing the final recipients to estrus simultaneously with the oocyte donor. The permanent recipients are anesthetized by intravenous barbiturate and gaseous anesthetics, the reproductive tract is exposed by mid-ventral laparotomy, and the oviduct or uterus is temporarily cannulated for transfer of the embryos.

Alternatively, three small puncture incisions are made anterior to the udder, and a laparoscope, manipulating forceps and needle are inserted for manipulation of the uterus. The oviduct or uterus is temporarily cannulated for transfer of the embryos, and the incision is sutured closed.

Recipients of oocytes with a targeted nucleus, engrafted in the manner outlined, were monitored for the status of their pregnancy by subcutaneous ultrasonic scanning on a weekly basis. For animals maintaining their pregnancy, the progress of the fetus is monitored regularly by ultrasound, and brought to term. Results are shown in Table 3. The longest-lived animal born with a PrP knockout survived 12 days.

TABLE 3: Nuclear Transfer from Gene Targeted Primary Cells

Stage of Animal Cloning	Nuclear donor cell		
	Parental Finn Dorset	α1,3GT targeted	PrP targeted
Reconstructions	126	142	454
Morula and blastocyst	33	21	43
Fetuses at day 60	5	5	8
Lambs at birth; live (dead)	0 (2)	0	3 (1)
Lambs alive at 1 week	0	0	1

Example 6: Gene targeting in telomerized fibroblasts

Primary Black Welsh fibroblasts (designation BW6F2) were transfected with the hTERT gene as described in Example 1. The characteristics of telomerized clone GRN1.1 are described in Example 2.

GRN1.1 cells at passage 5 or 25 were resuscitated into T175 flasks and grown to subconfluency. Cells (2.8×10^6, passage 5; 8.3×10^6, passage 25) were electroporated with 10 μg of NotI linearized p0054 targeting vector, using a setting of 125 μF: 350 V in Flowgen™ 0.4 cm / 800 μl cuvettes. Diluted cells were plated to 20 × 96 well plates. The next day, G418 (600 μg./mL) was added to the medium to begin the selection process. Cell death appeared after 8-10 days in G418, much longer than when using parental BW6F2 cells. Colonies were observed after ~2 weeks and replica plated (41 colonies from passage 5 cells; 2 colonies from passage 25 cells) on day 20 of selection.

Figure 6 shows results of PCR analysis on DNA isolated from the selected colonies. Lanes: 1-5 = primers for neo gene; 6-10 = primers for wild-type or targeted α1,3GT sequence. 1, 6 = B9 low DNA conc. (targeted). 2, 7 = B9 high DNA conc. (targeted). 3, 8 = C9 low DNA conc. 4, 9 = C9 high DNA conc. 5, 10 = 312 targeted positive control DNA. One targeting event (clone B9) was detected from the passage 5 electroporation.

This clone and eight non-targeted clones were resuscitated in 24 well plates. In all cases, cells grew to confluency. The B9 (correctly targeted) cell line, and the C9 cell line (one of the eight containing randomly integrated α1,3GT) grew fastest. Clones B9 and C9 have been karyotyped, and both are 54XY.

Thus, telomerized sheep fibroblasts were successfully targeted with the promoterless neo α1,3GT targeting vector, p0054. The targeted clone (B9) has been expanded, and retains a stable

karyotype. This clone exists as a pure population of targeted cells and continues to grow at passage 17 (~80 doublings). Successfully targeted clones can be used for nuclear transfer, or for targeting the same gene on the other allele, thereby creating a homozygous knockout.

Example 7: Targeting and telomerizing fibroblasts simultaneously

This example illustrates the design and use of targeting vectors that can be used to simultaneously inactivate an endogenous gene, and telomerize the cells for improved proliferation and genomic stability.

Figure 7 is a map of the two promoter-less-neo PrP gene knockout targeting vectors that were constructed. Both vectors have a 5' arm and a 3' arm of the sheep PrP gene. The 5' arm is a fusion DNA fragment with the 3' end of sheep PrP gene intron 2 and 5' end of exon 3. The 3' arm is the 3' end of sheep PrP gene exon 3. Contiguous with the sheep PrP 5' arm is the *neo* resistant gene whose transcription relies on the endogenous PrP promoter. Both vectors have the human telomerase gene (hTERT) inserted between PrP 5' and 3' arms and a phosphoglycerate kinase (PGK) promoter flanking 5' end of hTERT to direct its expression. The pPGK-hTERT gene in the two vectors is forward or reverse orientation, as indicated by arrows (forward for p115F and reverse for p115R). In addition, there are FRT site-specific recombinase sites on both sides of *neo* (for excision of *neo* gene with flp recombinase), plus aloxP sites on either side of *neo* and hTERT (for excision with Cre recombinase).

The p115R or p115F vectors were used to target sheep fibroblasts compared with the p0036 PrP targeting vector which does not contain hTERT. The results are summarized in Table 4.

TABLE 4: PrP Targeting Efficiency using hTERT Containing Vectors

Parental Line	PrP Targeting Vector	No. of G418 Resistant Colonies	Targeting Events Detected	Targeted Colonies Resuscitated
754F1 (Poll Dorset)	p115R (hTERT)	114	10 (8.8%)	7 (6.1%)
754F1 (Poll Dorset)	p115F (hTERT)	291	7 (2.4%)	6 (2%)
59F1 (Poll Dorset)	p0036 (no hTERT)	59	1 (1.7%)	0

Seventeen targeted cell clones were identified, of which 13 reached confluence after resuscitation. Overall, 3.2% of G418 resistant targeted clones were recovered without senescence. This contrasts to recovery frequencies below 1% using PrP targeting vectors without hTERT. Thus, including hTERT in gene targeting vector can increase the effective gene targeting efficiency by more than 10 fold.

Table 5 shows the karyotype analysis of five of the cell lines established from successfully targeted fibroblasts. Thirty cells were analyzed for each line.

TABLE 5: Karyotype of Targeted Cell Lines

	<52	52	53	54	55	56	>56	Conclusion
115F-C5p4	–	–	–	30	–	–	–	54 XX
115F-A1p4	–	1	–	29	–	–	–	54 XX
115R-D3p6	–	–	–	29	1	–	–	54 XX
115R-B2p5	1	2	3	24	–	–	–	54 XX
115F-G9p4	–	1	–	29	–	–	–	54 XX

These results show that in 4 of the 5 cell lines, virtually all the cells had the normal 54 autosomes characteristic of the sheep.

Figure 8 shows PCR analysis to identify cells in which the PrP gene was successfully targeted. The first primer hybridizes 5' to the PrP intron 2, and has the sequence TTCAGTCGC TCTGTTGTGTCCCA (SEQ. ID NO:29). The second primer hybridizes at the 3' end of PrP exon 3, and has the sequence AGCATCCC TCCTGCCTTC AGTTCTTC (SEQ. ID NO:30). Since intron 2 is deleted upon targeting, the second primer but not the first will hybridize where targeting is successful, and no amplification product will be produced (top panel). When the first primer is substituted with a sequence 5' to the *neo* gene (AGCCGATTG TCTGTTGTGC CCAGTCAT; SEQ. ID NO:31), amplification product should be obtained.

The results show that the PrP -neo band was amplified for the five targeted lines 115R-B2 (B2), 115R-D3 (D3), 115F-C5 (C5), 115F-G9 (G9) and 115F-A10 (A10), indicating homologous recombination with the targeting vector. Two PrP bands (3.9 and 4.6 Kb) amplified from L12, a cell line previously targeted with the p0036 vector which does not contain hTERT. As predicted, no PCR amplification took place using the *neo* primer in either the non-targeted cell line, 754F1-A1 (A1) or the parental cell line, 754F1 (F1).

Figure 9. shows Southern analysis of DNA prepared from resuscitated cells. The PrP probe was 450 bp in length amplified with a pair of PrP specific primers, 21F (CAAAAGAACT AGTTCCCC AATAAAC; SEQ. ID NO:31) and 21R (TAACAAATT TTACTTGCTGC TTGTG; SEQ. ID NO:33). The probe hybridizes to intron 2 of the PrP gene, showing a 6 kb BglI band in the wild type. When homologous recombination occurs, this BglI site is deleted and a fragment of 1.3 kb from neo-hTERT added. In cells targeted with either p115R or p115F, the probe detected a second band of 7.3 kb corresponding to the targeted allele.

Figure 10 shows the telomere restriction fragments (TRF) of targeted and non-targeted cell lines. After DNAs were digested and blotted, a labeled telomere repeat sequence was used to hybridize the blot. The result shows here, that all the targeted cell lines, 115R-B2 (B2), 115R-D3 (D3), 115F-C5 (C5), 115F-G9 (G9) and 115F-A10 (A10) as well as the early passage parental cell line, 754F1 (F1) maintained their telomere lengths. In most differentiated cells, the telomerase activity is not expressed and telomere length shortens with each cell division. Thus, both the non-targeted fibroblast line 754F1-A1 (A1) and

cells targeted without telomerization (L12) the telomeres shortened. Human genomic DNA (HG) was used here as control. Quantitation of TRF length is shown in Table 6.

TABLE 6: Characteristics of Targeted Colonies

Targeted Colony	Targeting Vector	Passage	Targeting Confirmed		Normal Karyotype	TRF
			PCR	Southern		
115R-B2	p115R	P5	+	+	80%	Long
115R-D3	p115R	P6	+	+	97%	Long
115F-C5	p115F	P4	+	+	100%	Long
115-G9	p115F	P4	+	+	97%	Long
754F1			–	–	100%	Long
754F1-A1		P4	–	–	97%	Short

The telomeres of cell lines simultaneously targeted and telomerized are the same length as early passage sheep fibroblasts — and (unlike non-telomerized cells) are maintained in proliferative culture. It is predicted that these cells are suitable for nuclear targeting with or without further genetic modification.

The compositions and procedures provided in the description can be effectively modified by those skilled in the art without departing from the spirit of the invention embodied in the claims that follow.

SEQUENCE DATA

TABLE 7: Sequences Listed in this Disclosure

SEQ. ID NO:	Descriptive Annotation	Species of Origin
1	Sequence of plasmid pGRN145, containing the human TERT sequence.	Artificial construct comprising human TERT, myeloproliferative sarcoma virus (MPSV) promoter, and vector components
2	α1,3GT cDNA sequence	Sheep
3	α1,3GT amino acid sequence	Sheep
4	PrP cDNA sequence	Sheep
5	PrP cDNA amino acid sequence	Sheep
6	Sequence of plasmid pWGB5a, in which the hTERT expression cassette is flanked on each side by a *loxP* recombination	Artificial construct comprising human TERT, myeloproliferative sarcoma virus (MPSV) promoter, and vector components

TABLE 7: Sequences Listed in this Disclosure

SEQ. ID NO:	Descriptive Annotation	Species of Origin
	recognition site	
7 to 33	Probes and PCR primers	Artificial sequences and sequence fragments

SEQ. ID NO:1

TGATC
ctctagagtcggtgggcctcgggggcgggtgcggggtcggcggggccgccccgggtggcttcggtcggag
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CLAIMS

What is claimed as the invention is:

1. A method for producing a vertebrate cell with several alterations in the genome, comprising:
   a) increasing expression of telomerase reverse transcriptase (TERT) in the cell;
   b) making an alteration to a particular gene in the cell or its progeny; before or after
   c) making a further genetic alteration to the genome of the cell or its progeny.

2. A method for producing a chimeric cell, comprising producing a cell with several alterations in the genome by the method of claim 1 and then transferring the nucleus of the cell with the altered genome to a suitable recipient cell.

3. A method for producing a non-human vertebrate, comprising producing a cell with several alterations in the genome by the method of claim 1, producing an embryo from the cell with the altered genome, and producing the vertebrate from the embryo.

4. The method of claims 1-3, whereby the gene is inactivated on at least one allele by homologous recombination.

5. The method of claims 1-4, whereby the gene is inactivated on both alleles.

6. The method of claims 1-5, whereby at least one allele of the gene is genetically altered to introduce a new encoding region in a manner that permits it to be expressed by the cell or its progeny.

7. The method of any of claims 1-6, further comprising separating cells in which one allele of the gene is inactivated using a first drug, and separating cells in which the other allele of the same gene is inactivated by selection using a second drug that is different from the first.

8. The method of claims 1-7, wherein the TERT is expressed transiently in the cell or its progeny.

9. The method of claims 1-8, wherein an encoding sequence for TERT is integrated into the genome of the cell or its progeny.

10. The method of claim 9, wherein the encoding sequence for TERT is placed under control of an endogenous promoter in at least one allele of the gene.

11. The method of claims 9-10, further comprising treating the cell or its progeny with a site-specific recombinase to remove the TERT encoding region from the genome of the cell.

12. A cell produced according to the method of claims 1-11, which is a nuclear donor cell.

13. An embryo produced from a chimeric cell prepared according to claims 2-11.

14. An embryonic stem cell produced from a chimeric cell prepared according to claims 2-11.

15. A non-human vertebrate animal, produced according to the method of claims 3-11.

16. A cell of a vertebrate animal cultured ex vivo, in which a normally expressed diploid gene contains a different artificially introduced genetic alteration in each allele.

17. A non-human vertebrate embryo in which a normally expressed diploid gene is altered by a different artificially introduced genetic alteration in each allele.

18. The cell or embryo of claims 16-17, the genome of which further comprises a recombinant nucleic acid encoding telomerase reverse transcriptase (TERT).

19. A non-human vertebrate animal in which a normally expressed diploid gene is altered by a different artificially introduced genetic alteration in each allele.

20. A vertebrate cell line genetically altered for increased expression of telomerase reverse transcriptase (TERT), which has been selected for high relative frequency of successful genetic targeting and is suitable as a donor for nuclear transfer.

21. The cell line of claim 20, wherein the frequency of successful genetic targeting by homologous recombination using a vector comprising at least 2 kb of DNA sequence isogenic to the host is at least ~0.2%.

22. The cell line of claim 21, bearing a TERT encoding region in the genome flanked by recognition sites for a site-specific recombinase.

23. A vector for homologous recombination in a eukaryotic cell, comprising a telomerase reverse transcriptase (TERT) encoding region.

24. The vector of claim 23, wherein the TERT encoding region is flanked on either side by recognition sites for a site-specific recombinase.

25. The vector of claims 23-24, wherein homologous recombination of the vector into the eukaryotic cell renders an endogenous gene in the cell inactive.

26. The vector of claim 25, wherein the endogenous gene is a PrP gene.

27. The vector of claims 23-26, further comprising an antibiotic resistance gene that can be excised using a different site-specific recombinase.

28. The vector of claim 23-26, wherein homologous recombination of the vector into the eukaryotic cell causes TERT to be expressed under control of an endogenous promoter.

29. The vector of claim 23-28, wherein the TERT is human telomerase reverse transcriptase.

30. The cell, embryo or animal of claims 17-20, wherein expression of a protein encoding sequence of the gene is inactivated on both alleles.

31. The cell, embryo or animal of claims 17-21, wherein each allele of the gene comprises a different artificially introduced nucleic acid sequence.

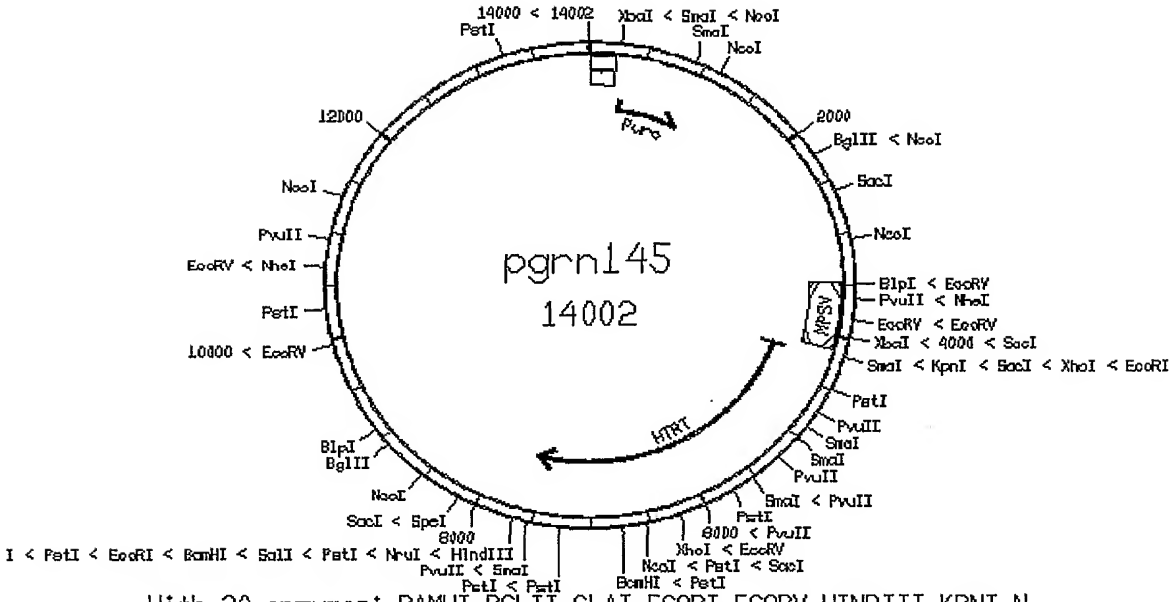
32. The cell, embryo, animal, or method of claims 1-19, wherein the gene is a prion protein (PrP) gene.

33. The cell, embryo, animal, or method of claims 1-19, wherein the gene encodes a glycosyl transferase.

34. The cell, cell line, embryo, animal, or method of any preceding claim, wherein the vertebrate is a livestock species.

35. The cell, cell line, embryo, animal, or method of claim 29, wherein the vertebrate is ovine, porcine, or bovine.

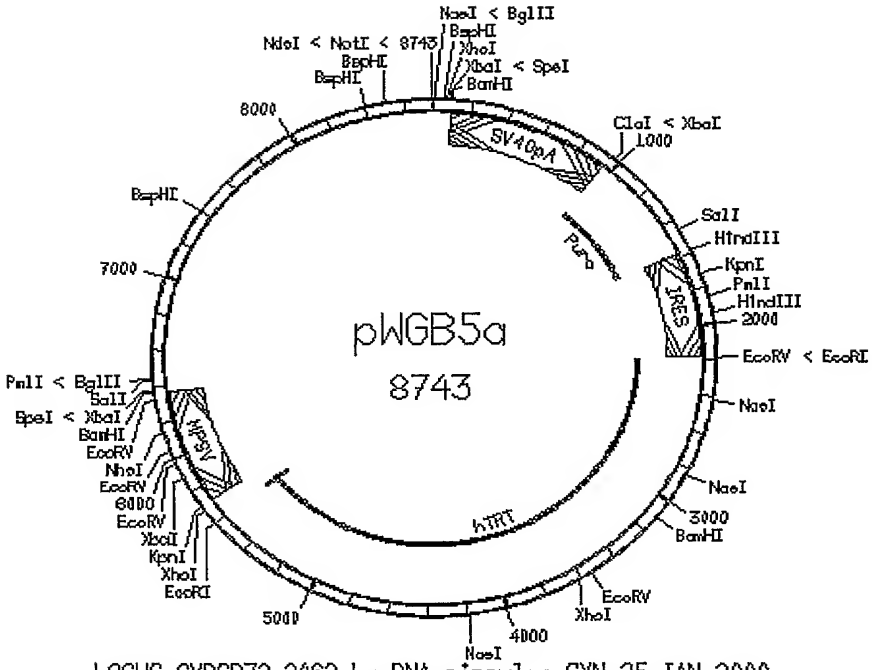
Figure 1



With 20 enzymes: BAMHI BGLII CLAI ECORI ECORV HINDIII KPNI N

February 8, 2000 15:08

Figure 2



LOCUS CVPSP72 2462 bp DNA circular SYN 25-JAN-2000
DEFINITION Cloning vector pSP72.
ACCESSION X65332

Figure 3

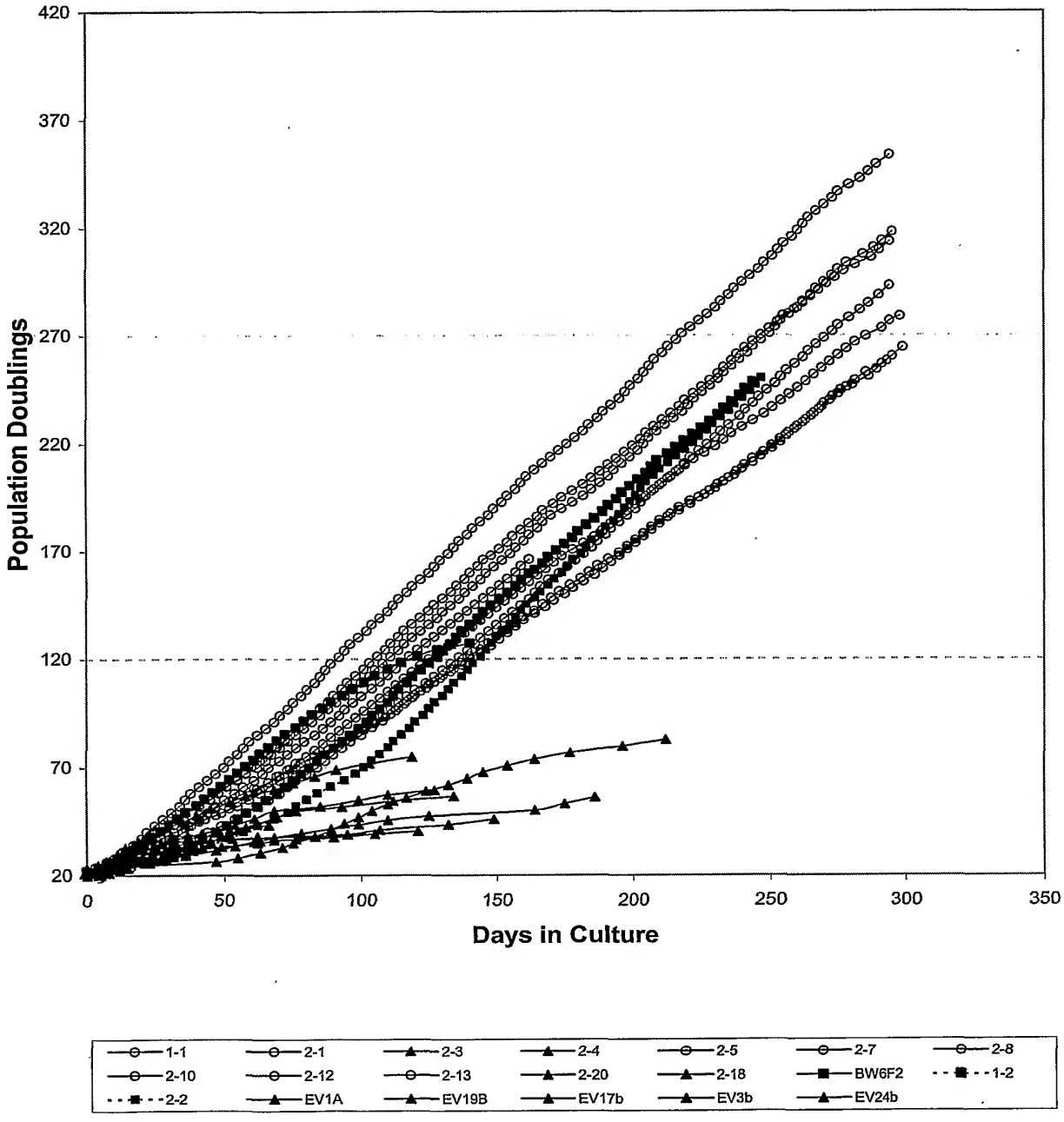

Population Doublings
420
370
320
270
220
170
120
70
20
0
50
100
150
200
250
300
350
Days in Culture
1-1
2-1
2-3
2-4
2-5
2-7
2-8
2-10
2-12
2-13
2-20
2-18
BW6F2
1-2
2-2
EV1A
EV19B
EV17b
EV3b
EV24b

Figure 4

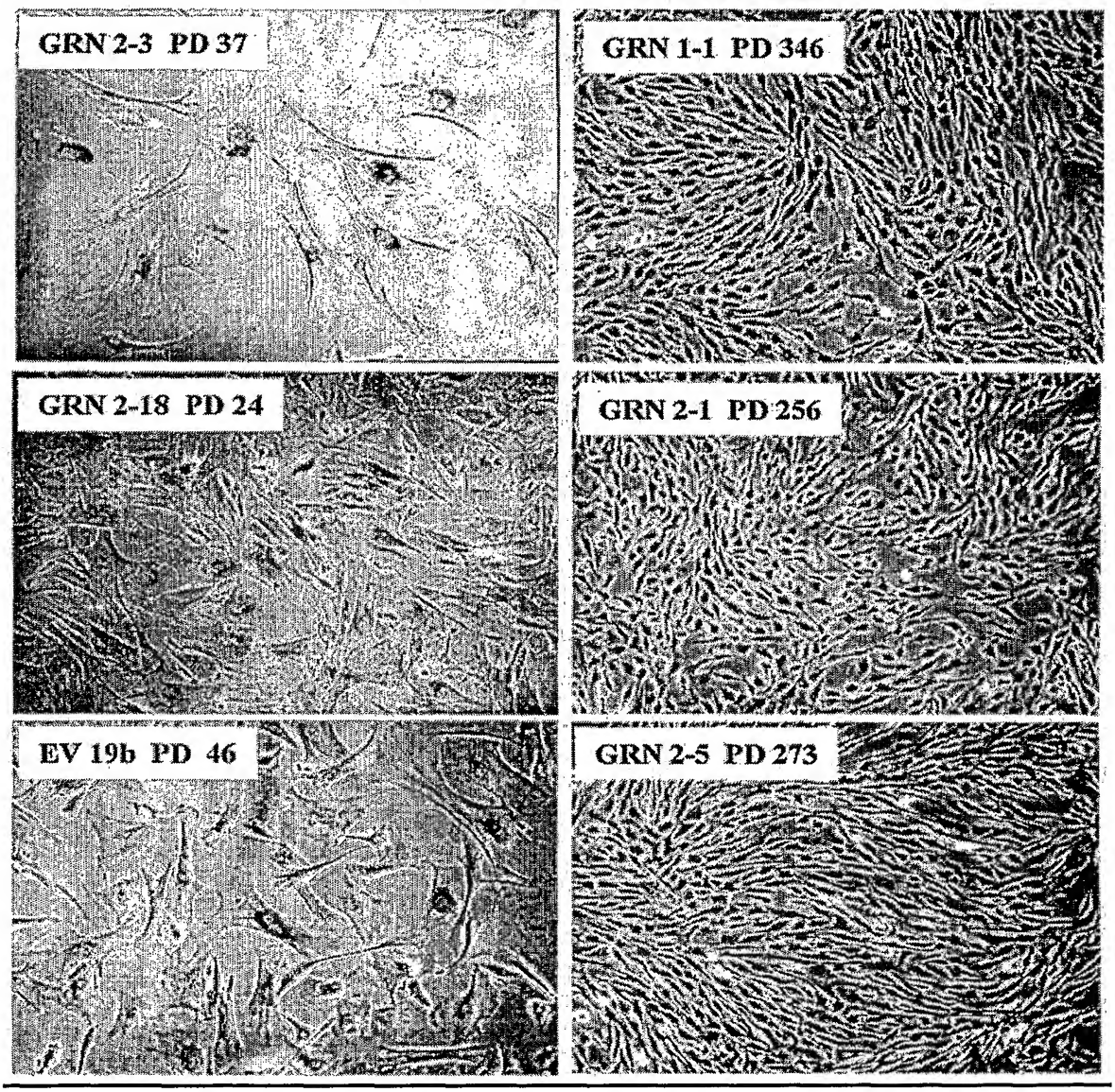

GRN 2-3 PD 37
GRN 1-1 PD 346
GRN 2-18 PD 24
GRN 2-1 PD 256
EV 19b PD 46
GRN 2-5 PD 273

Figure 5

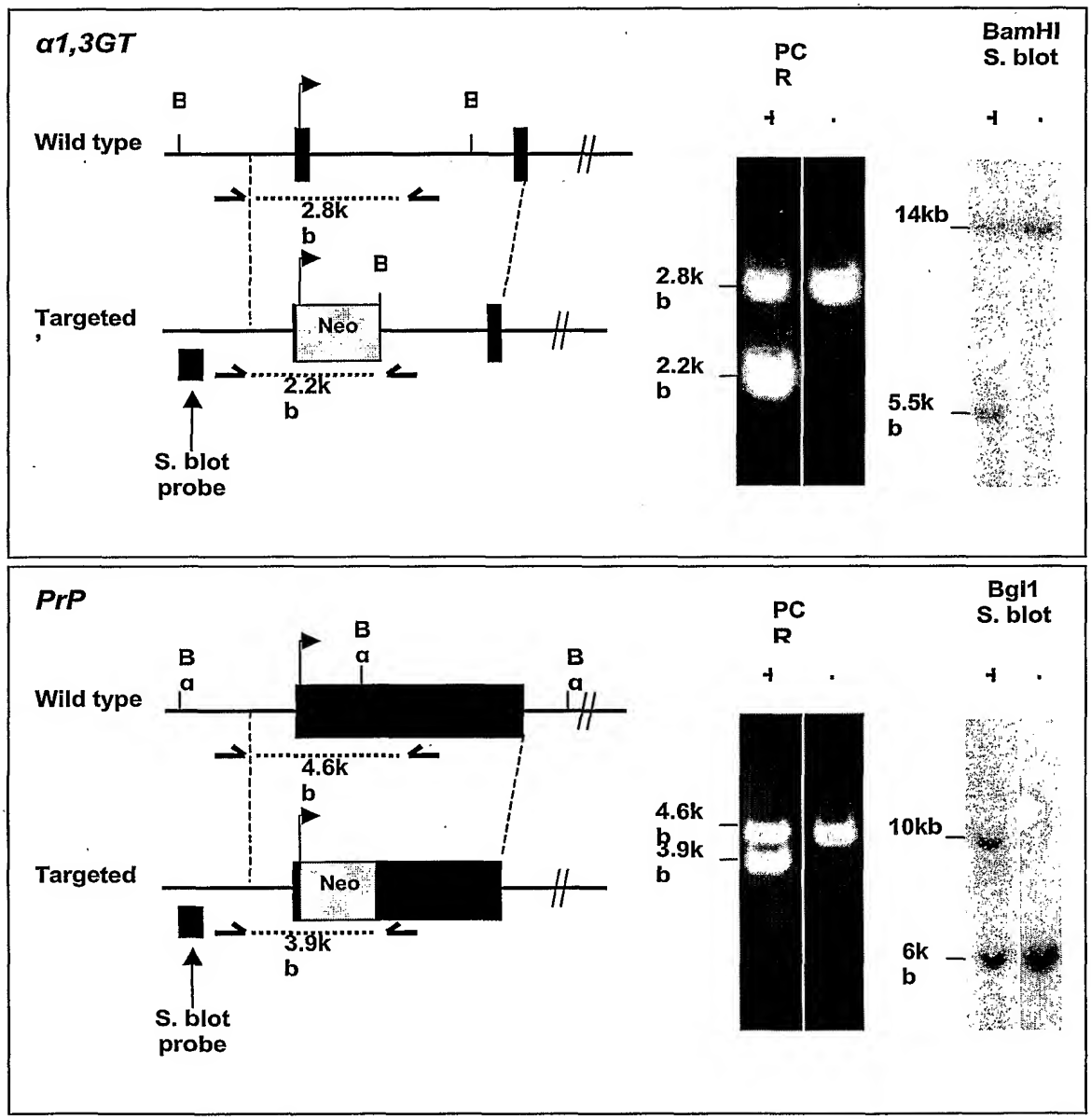

α1,3GT
B
B
Wild type
2.8k b
B
Targeted
Neo
2.2k b
S. blot probe
PC R
2.8k b
2.2k b
BamHI S. blot
14kb
5.5k b
PrP
B α
B α
B α
Wild type
4.6k b
Targeted
Neo
3.9k b
S. blot probe
PC R
4.6k b
3.9k b
Bgl1 S. blot
10kb
6k b

Figure 6

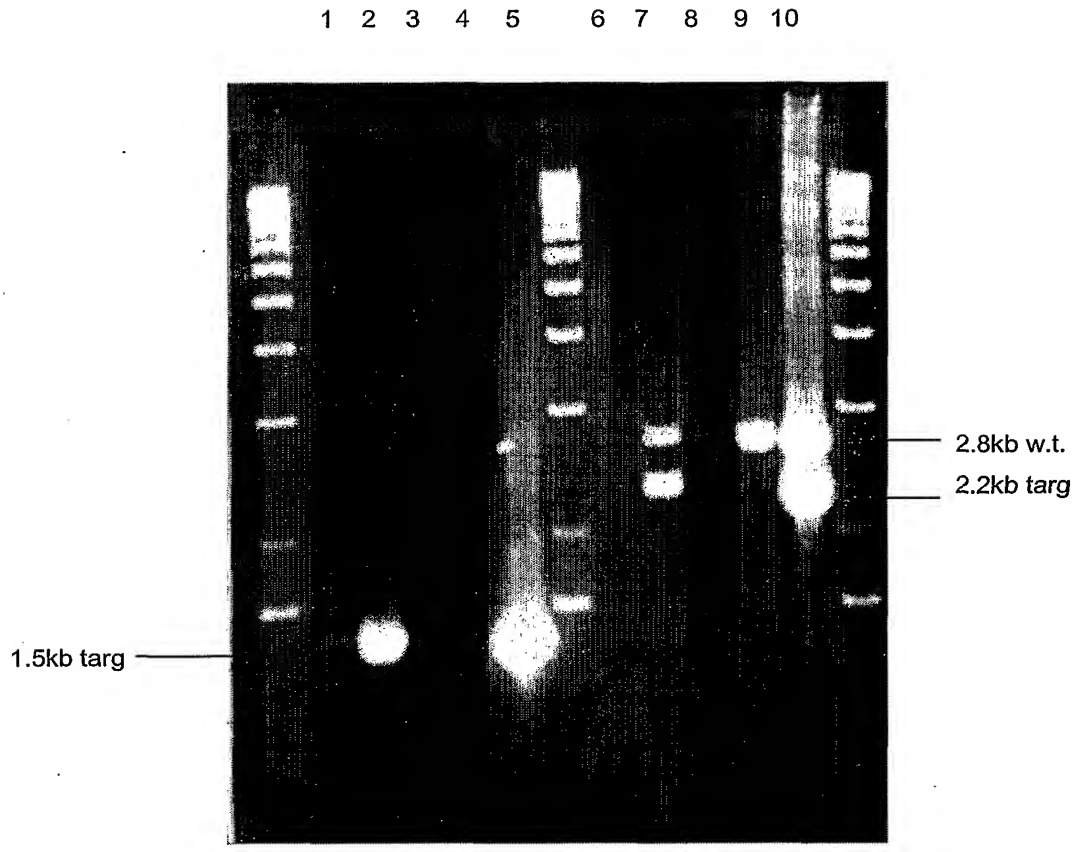


Figure 7

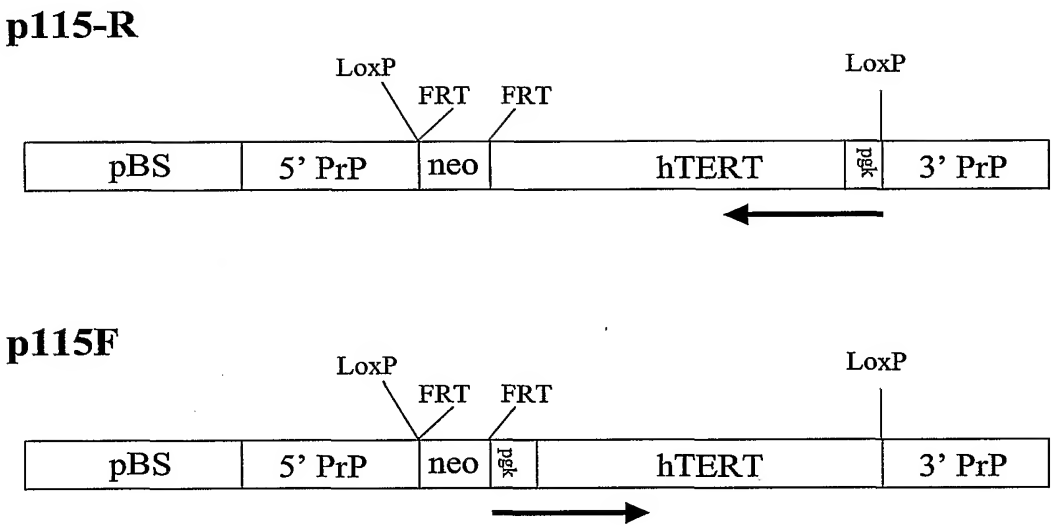


Figure 8

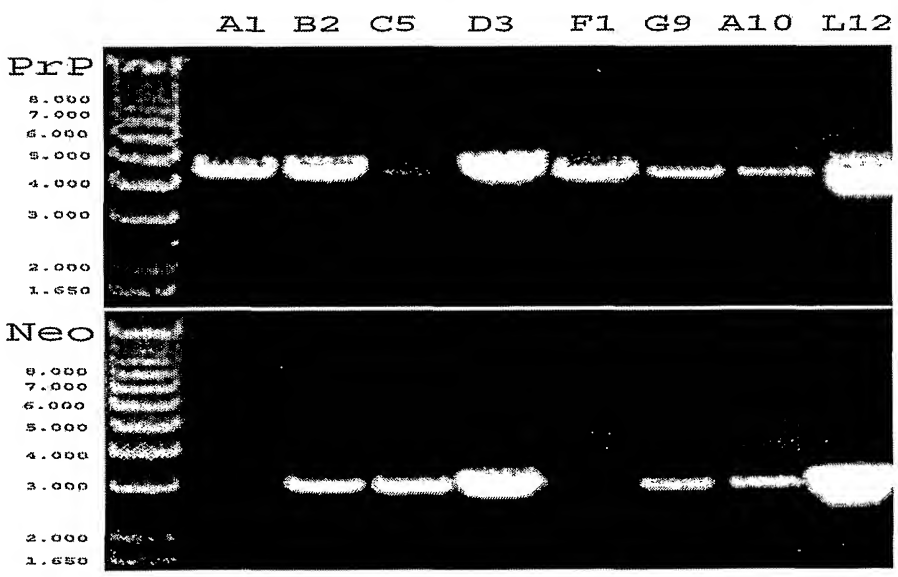


Figure 9

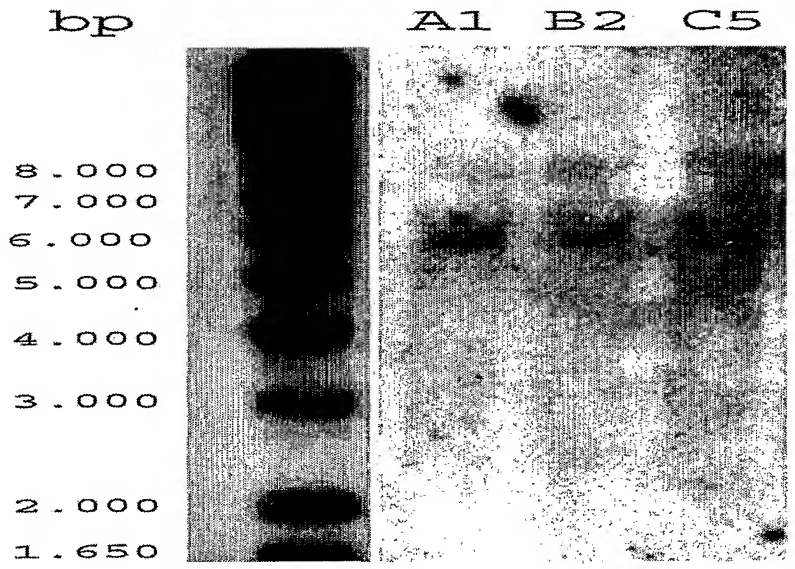


Figure 10